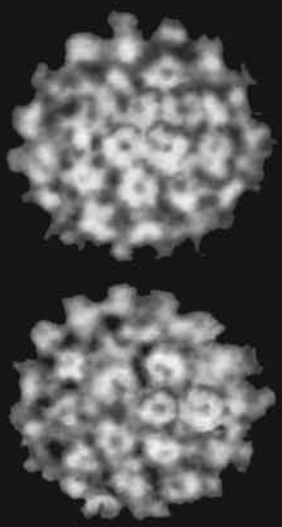
Role of tumor suppressors in the pathogenesis and treatment of squamous skin tumors in renal transplant recipients



Willeke A.M. Blokx

Role of tumor suppressors in the pathogenesis and treatment of squamous skin tumors in renal transplant recipients

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen.

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door

Wilhelmina Antonia Maria Blokx
geboren 30 juni 1967 te Den Dungen

PROMOTORES: Prof. Dr. D.J.Ruiter
Prof. Dr. Dr. P.C.M. van de Kerkhof

CO-PROMOTORES: Dr. E.M.G.J. de Jong
Dr. P.C.M. de Wilde

MANUSCRIPTCOMMISSIE: Prof.Dr. J.H.M. Berden
Prof.Dr. A.H.M.Geurts-van Kessel
Prof.Dr. Th.Starink, VU Medisch Centrum Amsterdam

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Chapter 1

GENERAL INTRODUCTION

SUMMARY
This thesis deals with new insights on the role of tumor suppressor proteins p53, p16, and p14 in (pre)malignant epidermal tumors of renal transplant recipients (RTRs) and immunocompetent individuals (ICIs). Expression of these tumor suppressor proteins in relation to important etiologic factors in cutaneous carcinogenesis, such as sun exposure, immune status, and Human Papilloma Virus are studied in epidermal skin tumors of both patient groups. Furthermore, the use of mutation analysis for p53 and INK4a-ARF is investigated in case of multiple primaries in patients with metastatic cutaneous squamous cell carcinoma.
The last part of the thesis deals with systemic retinoid treatment in skin (pre)cancer in RTRs. Retinoids are known to influence proliferation and differentiation. The effects of systemic retinoids on expression of markers for differentiation, proliferation and tumor suppressor protein expression are studied in these transplant recipients.
The introduction comprises a review on cutaneous squamous cell carcinogenesis in renal transplant recipients, and on the role of the tumor suppressors p53 and INK4a-ARF in cutaneous carcinogenesis. The main features of systemic retinoid treatment in skin malignancies will be reviewed.
These three topics will each be discussed in a separate part of the introduction.

PART I

SQUAMOUS CELL CARCINOGENESIS IN RENAL TRANSPLANT RECIPIENTS

1.1.1
Introduction

Transplant recipients have an increased incidence of cancers that arise de novo after transplantation. The prevalence in several large series ranges from 4% to 18%, with an average of 6% [149].
In transplant recipients a variety of uncommon tumors, such as Kaposi sarcoma, occurs which are seldom encountered in the general population. Of the malignancies that are also frequently seen in the general population, cancers of the skin and lips are the most commonly encountered de novo cancers in organ allograft recipients comprising 37% of all newly presented tumors. Within the group of organ allograft recipients, the largest group of patients is formed by renal transplant recipients (RTRs). Since the incidence of skin cancer increases with the number of years after transplantation, skin cancers are especially a problem in these RTRs because of their longer survival after transplantation when compared to for instance heart transplant recipients.
At present, RTRs account for a still growing patient population visiting the dermatology department and attribute significantly to the workload for the dermatopathologist in hospitals, harboring a renal transplantation center. The multiplicity of skin cancers in these patients, their more aggressive behavior and also the difficulties in clinical assessment of skin lesions in these RTRs leads to frequent and often multiple biopsies of clinically suspect skin lesions.
Several large epidemiological studies have been performed in the past which all implicate sun exposition, immunosuppressive treatment, and Human Papilloma virus as important etiological factors. However, until now, the exact mechanism for the enhanced cutaneous carcinogenesis in this specific patient group is still unknown.
Histopathological, immunohistochemical and molecular pathological studies of skin lesions in RTRs can be expected to yield important mechanistical information on the pathogenesis of skin cancer in these patients.
In this part, the epidemiology and etiology of skin (pre)cancer in RTRs will be addressed, with focus on squamous cell neoplasia.

1.1.2
Epidemiology of skin cancer in RTRs

Warts and keratotic lesions are the most frequently encountered epithelial neoplasms in RTRs [30]; they affect up to 48% to 100% of the renal transplant recipients [5,6,8,50,171] and are localized on sun-exposed areas, such as the face, forearms and back of the hands. When compared to the general population the warts are more numerous and more resistant to therapy. RTRs with high sun exposure are found to have significantly more warts than those with normal sun exposure [30]. The incidence of warts increases with time after transplantation; Barr et al. found warts in 20% of RTRs with graft survival below 5 years, compared to 77% in patients with graft survival of 5 years or more. Bouwes Bavinck et al. found a comparable mean interval from transplantation to the development of warts of 6.6+/-3.6 years. Warts preceded the development of the first skin cancers by 2-3 years [8]. Ramsay et al. found presence and large number of viral warts associated with SCCs risk and numbers [154]

Figures on the incidence of actinic keratoses (AKs) in renal transplant recipients are scarce and most are based on relatively small groups of RTRs with diagnosis of AK often only based on clinical grounds without histological confirmation. Boyle reported an incidence of AKs of 7.4% in 94 RTRs with lesions being confined to recipients with a previous history of high sun exposure [30]; Seckin described an incidence of 5% for AKs in 80 RTRs [171]; in both studies there was a short mean follow-up period of less than 5 years. Barr et al. found up to 38% keratoses in RTRs with graft survival above 5 years, although they often experienced difficulties in clinically separating keratoses and viral warts [6].

In the past 3 decades several often large retrospective studies[16,26,82,86,94,117,154,210] and reviews [11,50,60,64,111] on the incidence and pathogenesis of skin cancer in RTRs and organ transplant recipients have been performed and published (**Table 1A)**.

These studies and reviews yield the following features characteristic for cutaneous carcinogenesis in RTRs:

1. There is an increase in the overall incidence of skin cancer, both squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), when compared to the general population, in organ and renal transplant recipients.
2. The increase in incidence of SCC by far outnumbers the increase in BCCs, and as a consequence a reversal of the BCC:SCC ratio is seen, varying from 2:1 to 3:1,when compared to the general population in which BCCs outnumber SCCs by a ratio of 5:1 [64,149]. The relative risk of developing carcinomas seems to increase linear for BCCs, and exponentially for SCCs [210].
3. The overall risk to develop skin cancer increases with time after transplantation, affecting half of the patients within 20 years posttransplantation [26,64,210]. In the Netherlands, a 770-fold rise of incidence of SCC was found in RTRs with more than 15 years graft survival, while the risk in the group of RTRs as a whole was 250-fold increased compared to the general population [82].
4. SCCs in RTRs tend to be multiple. About half of the patients have more than one SCC and one third also has BCCs [63]. Carcinomas are usually associated with warts, premalignant keratoses, Bowen's disease and keratoacanthomas [179].
5. SCCs are more aggressive than in non immunosuppressed patients. In 12% local recurrences and in 8% metastatic disease occur [64]. The primary site of metastasis is the lymph node, as in the general population [125].

TABLE 1A Studies on the incidence of skin cancer in RTRs

Ref.	year	country	no.	follow-up (mean yrs PT)	vv %2	ak %	mb %	bcc %	scc %	remarks
184	1977	U.S.A.	584	3.3+/-0.5				0.5	1	
81	1980	Australia	290							7.5% developed NMSC. 20.6 times increase NMSC incidence. Ratio BCC:SCC=1:1.7. Often multiple skin cancers. First skin cancer after mean interval of 34 mo.
30	1984	U.K.	94	4.5	31	7			2	
115	1985	U.K.	108			9.2			4.6	Mean interval of 5.1 yrs between transplantation and development skin lesions
179	1987	U.K.	85	6.8	53	28				
101	1987	Australia	73	7.8-12.7				18	36	
31	1988	Ireland	330	6.6	24	21		0.6	2	4 fold increase NMSC (BCCs included)
133	1988	U.K	121	5.9	37	6	6	2.5	2.5	Warts if present often multiple.
6	1989	Scotland	202	< 5 yrs	20	17				Ratio SCC : BCC = 15 : 1
				> 5 yrs	77	38				
82	1990	NL	764	8.7				5	18	Overall risk for SCC 253 times higher than general population. Risk for BCC only 10 fold increased incidence
159	1992	Austr-NZ	2115						6	
24	1993	NL	137	13.1				12	21	
12	1993	Sweden	173		40	10	1	8	2	13% multiple warts. 25% of patients with tumors had multiple warts.
77	1994	U.K.	291	4.8	59			12	14	No skin cancer in brown or black skin .
16	1995	Scandin.	5.692							18-29 fold increase NMSC (BCCs not included)
63	1995	France	580			54	36	35	47	Ratio SCC:BCC=2.4:1 Heart transplant recipients 2-fold increase in epithelial neoplasia compared to RTRs.
26	1996	Australia	1.098	12				19	31	45% skin cancer risk after 11 years 70% skin cancer risk after 20 years
5	1997	Italy	199	6-13	37		1	2		
86	1997	France	1700	4.3-7.6						Skin cancer risk after 10 yrs 1.83-7.14% with highest skin cancer risk for patients on triple or multiple immunosuppressive therapies .
210	1997	Turkey	1069					2	5	Non-Caucasians developed no NMSCs
51	1998	France	150	2.2	28					
171	1998	Turkey	80	4.0	36	5		3	1	
195	1999	Italy	423	7.6			1	2.4	1.4	
94	1999	Norway	2397	4.8					3.6	60-fold increased risk for SCC
40	1999	U.S.	122	3.1	25			6.5	3.3	
155	2000	U.K.	182	8.5+/-6.3	53	15	13	4	6	Ratio SCC:BCC =3.8:1 Increase warts, AK and NMSC with interval PT
124	2000	U.K.	222	> 5 yrs				6	11	

6. The distribution of SCCs differs considerably from that of BCCs. SCCs occur on sun-exposed areas with a preference for the backs of the hands and the face, while BCCs frequently occur on the upper chest but not on the back of the hands [82]
7. There is a strong race effect with series from Western countries only mentioning Caucasian recipients being at risk for developing non-melanoma skin cancer (NMSC) after transplantation [77,210].
8.Transplant recipients are affected by NMSCs at an average age 30 years below that of the general population in which NMSCs mainly occur in people in their 60s and 70s [149].
9. The percentage of transplant recipients with skin cancer increases with decreasing latitude. In Australia 45% develop skin cancer within 10 years after transplantation, compared to 10-15% in for instance the Netherlands [82].
The number of patients with premalignant and malignant epidermal cutaneous neoplasms shows considerable variation between the studies listed in **Table 1A**. This is attributable to differences in mean follow-up of patients, variability in the UVB exposure of the investigated populations, changes in long term immunosuppressive therapy, rejection treatment, and in development in HLA-matching of donors with recipients. The latter three factors can be expected to have a continuing impact on the skin cancer incidence in these patients and demand up-to-date cancer registration in RTRs.

In conclusion, these epidemiological data indicate that skin problems and especially skin cancer is a major cause of morbidity and even mortality in the population of RTRs. Insight into the pathogenesis of the enhanced cutaneous carcinogenesis in these patients might attribute to better future preventive and therapeutically treatment modalities.

1.1.3
Etiological and risk factors of skin cancer in RTRs

1.1.3.1 Sunlight and UVB exposure

Epidemiologic evidence

Exposure to sunlight is believed to be one of the most important risk factors for the development of both warts and skin cancers in RTRs as suggested by the location of warts and carcinoma on sun-exposed areas of Caucasian transplant recipients [6,30,82,133]. In the non-immunosuppressed population sunlight is also a major factor in the development of non melanoma skin cancers, but in contrast to renal recipients, relation between sunlight exposure and development of warts in immunocompetents has not been reported.

Several investigators have compared the incidence of skin cancer in transplant recipients with high and low sun exposure and they all showed an increase of skin cancer in the first. For instance, Bouwes Bavinck et al. reported a relative risk of 4.3 for developing skin cancer after renal transplantation in Queensland, Australia, when compared with the Netherlands: after 20 years of immunosuppression in the Australian cohort the cumulative skin cancer incidence increased to 70%, while in the Dutch patients the incidence 20 years post-transplantation increased to 41% [26,82]. In the Dutch population 97% of the SCCs occurred on sun-exposed areas of the skin. In another separate study in Dutch renal transplant recipients, exposure to sunlight before the age of 30 was found to contribute more to the risk of skin cancer development than exposure after the age of 30 [8]. Barr et al. studied 202 renal allograft recipients in Scotland and in all but 1 of the 10 recipients with skin cancer, sun exposure was high. Furthermore all cancers occurred on sun-exposed skin [6].

Boyle et al. reported only AKs and SCCs in RTRs with a history of high sun exposure [30].

Mutagenic effects of ultraviolet radiation

Skin cancers are due to a complex of simultaneous and sequential biochemical events, caused, and promoted by UV-radiation of varying wave length (UVB 280-320nm, UVA 320-400nm, and perhaps visible light and infrared). These radiations produce DNA lesions, that may if not repaired lead to mutations in proto-oncogenes and tumor suppressor genes [168]. UVA gives rise to lesions probably by production of oxygen activated species (ROS), while UVB (main factor in sun exposure) is directly absorbed by DNA.

UVB exposure induces predominantly DNA lesions between two adjacent pyrimidine bases (TT, CT, TC, CC) on the same DNA strand, forming essential 2 mutagenic dimers (cyclobutane-pyrimidine dimers and (6-4) pyrimidine-pyrimidones) between these bases, that give rise to mutations mainly characterized by C to T or CC to TT transitions always located at the sites of pyrimidine-pyrimidine sequences [42,54,168]. These mutations are now considered as a true signature of sun exposure. The CC to TT tandem transitions is absolutely specific of UV radiation.

The pyrimidine dimers caused by solar DNA damage, can enzymatically be repaired by nucleotide excision repair (NER) [74,168]. Xeroderma Pigmentosum (XP) patients lack this type of repair and have a strongly increased risk of skin cancers [42].

UV-type mutations have been found in for instance p53, more recently in INK4A-ARF, ras oncogenes, and PTCH tumor suppressor genes [74,168,219]. The first two will be discussed in more detail in PART II of this thesis.

In NMSC the p53 gene appears to bear point mutations with features of UVB induced mutations, i.e., association with di-pyrimidinic sites, mostly C to T transitions and 5-10% CC to TT tandem mutations [42].
Giglia-Mari et al. analyzed mutational hot spots in skin cancers, malignant melanomas, and NMSCs. One hot spot was common to all skin cancers, the Arg248. In skin SCCs a hot spot at codon 278 seems specific [74].

Immunosuppressive effects of UV
Ultraviolet radiation might predispose to skin cancer by down regulation of local immune response of the skin. Langerhans' cells (LCs) are a major component of the skin's immune system. They are a population of antigen presenting cells that recognize and transport antigen to the local lymph node where it is presented in a processed form to antigen specific T-lymphocytes and it has been suggested that they participate in immune response to viral and tumor antigens [156]. The skin must have an adequate density of Langerhans' cells otherwise an immune response is not generated.
As reviewed by Meunier, UVB has been shown to deplete epidermal Langerhans' cells [135]. After severe sunburn, LCs are replaced by circulating bone marrow derived precursors and by DCs migrating from hair follicles that have a partial deficiency of molecules important for T cell costimulation.
Mechanisms underlying the LC depletion after UVB radiation are still unknown. Chronic UVB radiation may cause deficiency of growth factors for LCs or abrogate cytokine responsiveness of DCs by down regulating the expression of surface receptors for growth factors. UVB radiation may trigger apoptosis of resident epidermal LCs, or cause LCs to migrate to the lymph node. UVB irradiation of human LCs decreases their capacity to induce proliferation of CD4 and CD8 T cells. Furthermore UVB inhibits the antigen presenting capacity of LCs by interfering with antigen processing [135]
Some investigators found marked depletion of Langerhans' cells in benign, premalignant and malignant skin lesions of RTRs when compared to normal skin [72], although no significant differences in Langerhans' cell numbers could be demonstrated between normal skin of RTRs and immunocompetent controls.
Galvao et al. found in transplant recipients a decrease in CD4 and CD8 positive T lymphocytes in exposed and non-sun exposed skin in the subset of the RTRs with a long interval post-transplantation [69]. In RTRs the CD1a+ dermal cells were reduced in sun-protected skin, and the CD1a epidermal cells were reduced in number and had small dendrites in sun-exposed skin, when compared to controls [69].

In conclusion, both epidemiologic data as well as molecular data on UV type mutations in skin cancers indicate that sun exposition is a major factor in the development of skin cancer in both RTRs and ICIs. In addition UVB might act carcinogenic by lowering local skin immunity by causing Langerhans' cell depletion.

1.1.3.2 Immunosuppressive treatment

Immunosuppression can increase carcinogenesis by direct carcinogenic effect of the immunosuppressive agents, or by creating a state of decreased immune surveillance and insufficient eradication of malignant cells. Direct carcinogenic effects are reported for azathioprine and cyclosporine as reviewed by Berg et al [11].
Clinical studies comparing azathioprine and cyclosporine based regimens yield conflicting results with respect to effects on skin cancer incidence. Some report greater proportion of skin cancers among azathioprine–treated patients, explained by its photosensitizing effects [11,150,154,181], while others found no differences [95], or a reduced risk compared to regimen which included cyclosporine [159]. Studies on newer medications such as tacrolimus suggest that the skin cancer risk continues to exist, but need to be established in the future since skin cancers take several years after transplantation to develop [11].
The incidence of skin cancer is correlated to immunosuppression levels.
Comparative epidemiologic study of premalignant and malignant epithelial neoplasia in kidney and heart transplant recipients implicate that the effects of immunosuppressive treatment are dosage dependent. Heart transplant recipients, who generally receive higher levels of immunosuppression, develop skin cancers earlier after transplantation and at a 2-3-fold higher rate when compared to RTRs [63].
Several studies have shown that patients on triple or multi-drug therapy have a higher cumulative risk for developing SCC than RTRs on double therapy [86,94,212].
A 5–year randomized, prospective study showed that low-dose cyclosporine regimen was associated with fewer malignant disorders, though rejections were more frequent [41].

In conclusion, skin cancer rate in RTRs is influenced by type and degree of immunosuppressive treatment. The continuing development in immunosuppressive treatment modalities, can expect to have impact on skin cancer development in these patients. Search for immunosuppressive treatment, which causes less secondary tumor development post transplantation, is of great importance for the group of RTRs being at risk for morbidity and mortality due to these secondary cancers.

1.1.3.3 Human Papilloma virus

Virology and Pathology of HPV infection

Human Papilloma viruses (HPVs) are increasingly recognized as pathogens in the development of specific human cancers. For instance, HPV is strongly implicated in the development of human uterine cervical cancer [53,85,192]. HPVs are non-enveloped, double stranded DNA viruses. Molecular cloning of viral nucleic acids and more recently polymerase chain reaction (PCR) amplification and sequencing have demonstrated that multiple HPV types exist with more than 100 HPV types now described [151,192]. HPVs can be classified on the base of specific tissue tropism, resulting in two main HPV groups: cutaneous and mucosal [151,192,201]. The cutaneous HPVs are found in cutaneous warts (HPV-1 in plantar warts and HPV-2 and 4 in common warts), and there is a large group of 20 or more HPV types that are associated with the rare disease Epidermodysplasia Verruciformis (EV), namely HPV types 5,8,9,12,14,15,19-25,36-38 and 47) [25]. The mucosal HPVs predominate in cervical and anogenital lesions, like condylomata accuminata, and are broadly classified into those with a low risk (HPV-6 and 11 predominantly) and those with a moderate-to-high risk for cancer development (HPV-16 and 18) [85,192].

All HPVs contain 2 functional groups of genes, the early genes that function primarily in episomal replication (E1 to E7) and late (L1 and L2) genes that encode the viral capside proteins [4,36,85].

HPVs are strongly epitheliotropic viruses and can be found in all human squamous epithelia and only basal replicating cells can be infected by HPVs. How the virus gains access to basal epithelial cells is not known and a viral receptor on host cells has not been identified to date, though recent data suggest that integrin complexes containing $\alpha 6$ integrin complexed with either $\beta 1$ or $\beta 4$ integrins may act as a receptor for papillomavirus binding and entry in epithelial cells[65].

Interestingly, although host-cell infection must occur in undifferentiated dividing (basal) cells, viral replication can only occur in cells committed to differentiation. Therefore in benign lesions only a low number of viral genomes are present in basal and suprabasal layers. Amplification of viral genomes occurs in stratum spinosum and granulosum with a high number of HPV copies in the superficial epithelial layers. It is in these terminally differentiated cells, that HPV gene expression (L gene expression) and protein synthesis (capsid protein expression) lead to histological visible cytopathogenic effects of HPV in epithelial cells with koilocytosis, nuclear enlargement, dyskeratosis and multinucleation being the most prominent [192]. In benign lesions, the viruses are episomal, located outside the host chromosomes, and eventually shed at the surface. In contrast to this permissive non-transformable HPV infection, HPV infection can also be non-permissive and transformable; in this situation viral replication and vegetative viral production do not occur, but viral DNA persist within the cells either as an extra-chromosal element or by integration into the host genome [4,85,98,192]. This persistence in cells is strongly associated with cellular transformation and implicated in the genesis of cervical dysplasia and cancer. Within the group of mucosal HPVs, especially HPV types 16, 18, 31 and 33, are classified as high risk viruses with respect to cervical cancer development [93].

Among viral infections in the epidermis, HPVs are of major importance [49]. Clinical infections (warts) are the most common in children and young adults, with an estimated incidence of 10%. The exact prevalence in the adult population is not known, however serological and DNA hybridization techniques suggest that viral exposure at this age with subclinical and latent infection might be very common. Although some warts spontaneously regress, warts in some adults and immunocomprised persons can persist for years [49].

The earliest link between warts and skin cancer was noted in the rare genetic disorder EV[83]. In these patients widespread skin warts occur from an average age of onset of six years and SCCs develop on light exposed sites in 30-60% of affected patients by the fourth decade, and specific HPV types, predominantly HPV-5 and HPV-8 , are found in more than 90% of the tumors [83]. With respect to the co-occurrence of often multiple warts and SCCs, RTRs resemble these EV-patients.

Model for HPV-related cervical neoplasia

Approximately 80% of high-grade squamous intraepithelial lesions (HSILs) and cervical cancers contain a restricted spectrum of just four HPV types (16,18, 31,33 and 45) and these HPV DNAs become integrated into host chromosomes[4,192]. Integration typically occurs within the viral E1 or E2 genomes and disruption of these E1 or E2 genes allows for deregulated/enhanced expression of E6 and E7, which are consistently expressed in HPV associated cervical cancers[4,192]. The E6 and E7 oncoproteins of these so called high risk HPV types interfere with two important pathways involved in cell cycle control: the retinoblastoma protein (pRb) pathway (E7) and the p53 pathway (E6). The E6 protein inactivates the p53 tumor suppressor protein, the functional equivalent of mutational inactivation. P53 is a prime regulator of cell proliferation and has been shown to regulate programmed cell death or apoptosis in response to DNA damage. By abrogating p53-related effects on cell proliferation and apoptosis upon DNA damage, E6 promotes chromosomal instability and unrestrained cell growth causing malignant progression. The high risk E7 oncoprotein, can complex with pRb, leading to functional inactivation of pRb and release of an active host transcription factor E2F , causing enhanced cell proliferation[4,192]. Presence of high risk HPV in cervical cancers, leads through the respective actions of the two oncoproteins E6 and E7, to typical immunohistochemically detectable changes in the expression profiles of important tumor suppressor proteins involved in the above mentioned Rb and p53 pathways.

For instance p16, involved in the p16-CDK4/6-RB pathway (for more detail see part II), is strongly overexpressed in almost all HPV-related cervical cancers with preserved pRb immunoreactivity[99,100,167]. This seems to be an E7-related effect, causing an overexpression of E2F, which in turn induces the transcription of p16 [100].

HPV in cutaneous neoplasia

In contrast to cervical neoplasia, in skin tumors harboring HPV DNA, the steps of progression from benign lesions to malignancy are not known.

So far, the kinetics of HPV in the epidermis is not well understood, and despite in vitro studies the virus cycle of HPV in keratinocytes remains unknown. HPV infection probably starts in basal cells which are reached by infecting virus particles probably through skin defects and so far no HPV cell receptors have yet been identified [98], although alpha-6 integrin specifically expressed on basal keratinocytes is suggested to function as possible HPV receptor [65].

As described above , the viral proteins crucial for tumorigenesis in the uterine cervix are E6 and E7 oncoproteins of high risk HPVs. The mechanism of action of E6 and E7 of oncogenic EV HPVs is not known. It was shown that E6 oncoprotein does not degrade p53 protein. Only recently, in vitro studies, showed that HPV-38 (an EV-HPV type) E7 was able to inactivate the tumor suppressor pRb with induced loss of G1/S transition control, and that E6 and E7 of HPV-38 were able to increase the life span of human cultured keratinocytes. In contrast to E6 of HPV-16, E6 of HPV-38 was unable to promote p53 degradation [34]. Since p53 is often mutated in skin cancers, in contrast to HPV induced cervical cancers, these data might suggest that E6-mediated p53 degradation is unnecessary in skin carcinogenesis, since UV-radiation has already lead to p53 inactivation.

In addition, in skin HPV E6 might prevent apoptosis in a p53-independent manner. In fact, it was reported that E6 of HPV-5, 10 and 77 in vitro were able to target and promote proteolysis of bak protein, a pro-apoptotic protein. This E6 induced elimination of Bak protein, could lead to decreased apoptosis in UV irradiated cells which might enhance tumor formation [92].
Both E6 and E7 are expressed in EV cancers but integration of HPV-5 DNA into the host DNA is an exception and occurs only in metastases[121], while in cervical and anogenital HPV associated cancers HPV DNAs are integrated in the genome [192].

In conclusion, the role in which HPVs are involved in skin carcinogenesis needs further elucidation. The role of HPV in skin cancer is most likely different from the role of HPV in cervical cancers, with UV induced p53 mutation rather than p53 inactivation by E6 oncoprotein playing a role. This might be expected to be reflected in different immunohistochemical profiles for important tumor suppressors involved in the p53 and RB pathways, in HPV induced skin neoplasia compared to cervical neoplasia. Therefore, in the present thesis immune expression profiles of three important oncoproteins, p53, p16, and p14, in (pre)malignant skin lesions in relation to HPV and other risk factors will be studied.

Role of HPV in cutaneous neoplasia of renal transplant recipients
A variety of HPV types can be detected in benign, premalignant, and malignant proliferations of RTRs (**Table 1B**) . In RTRs the most frequent HPV types are benign cutaneous types 1,2,4, EV-associated HPV types or possibly potentially oncogenic cutaneous types (HPV types 5,8,9,12,14,15,17,19-25,36-38 and 47) and mucosal HPV types, partially benign (6/11) or potentially oncogenic (HPV types 16,18). In many instances more than one HPV type in a single lesion is found.
The listed studies show considerable variation in the prevalence and types of HPV detected. These discrepancies are likely to reflect the different detection methods used. The early studies employed DNA hybridization based techniques that tend to be less specific and sensitive than later methods based on PCR. Furthermore DNA was extracted from frozen or formalin fixed specimen and of the large number of known HPV types especially earlier studies tested only a few. Some studies were performed on small series of lesions.
Even with the introduction of PCR-based techniques for HPV-detection, discrepancies in data continue to arise. This is also likely to be a methodological phenomenon with different investigators using different primer sets. Meyer et al, compared 4 PCR assays for detection of HPV in SCCs and found that the rate of HPV-DNA positive specimen increased with addition of nondegenerate primers derived from HPV-5 and 8 to 69% compared to PCR assays using only degenerate primers for broad range HPV detection (50% HPV positivity) [136]. Furthermore an increasing number of novel HPV sequences have been detected in NMSCs from RTRs in the past few years using PCRs with consensus primers that target the gene region of the L1 capside protein of HPV[14,172,173].

TABLE 1B Studies on HPV detection in renal transplant recipients: OTR= organ transplant recipient, RTR= renal transplant recipient, VV=verruca vulgaris, AK= actinic keratosis, MB= Bowen's disease, KA= keratoacanthoma, SCC= squamous cell carcinoma, BCC= basal cell carcinoma, H= DNA hybridization based technique.

Ref.	Year	No of lesions	RTRs	Techn	HPV types	HPV presence in warts and epidermal neoplasia
33	87	30		H	5	3% in VV, 33% in SCC
198	87	48	36	H	1,2,3,4,5,6	90% in VV, 1 SCC containing HPV 2 and 4 20% of VV two HPV types
17	89	59	13	H	1,2,4, 5,8	50% in VV, up to 40% in AK, 44% in SCCs, 0% in BCCs
58	89	33	5	H	1a,2a,16,18,5	76% lesions HPV positive, with 56% containing potentially oncogenic types 16/18. Only 8% HPV 5 positive.
6	89	154	72	H	1,2,4,5/8	76% in VV, 43% in AKs, 25% in SCCs , and 0% in BCCs 18% skin lesions in RTRs HPV5/8 positive ; 50% with skin cancer, only 13.5% in benign lesions.
52	91		189	H	1,2,3,4,7,10,5,8,14,17	HPV DNA in 62% of warts in RTRs and 83% of the warts in ICIs. In both groups HPV 2 was most prevalent. No EVA- HPV types in both groups.
148	92	34	?	H	1a,2a,5,6a,11,16, 18.	82% in VVs, 10% in AKs, 69% in SCCs. HPV DNA in 39% of lesions with 71% containing several HPV types. Benign and potentially oncogenic types in VV and SCC.
206	92	32	15	H	1a,2a,5,16,18,33	60% in VVs (type 2), 25% AKs (type 1 and 2), 33% MBs, 25% SCCs (type 2 and 18), 16% BCCs (type 2)
205	93	27	?	H	1,2,5,6,11,16,18	50% in VVs (type 2), 33% in MBs, 25% in SCCs, 16% in BCCs
59	93	86	24	H	1a,2a,5,16,18	82% in VVs, 24% in AKs, 25% in MBs, 75% in KAs, and 47% in SCCs
186	93	62	22	H PCR	1a,2a,5,6a, 11,16,18	61% in VVs, 58% in AKs, 66% in MBs, 0% in KAs, 87% in SCCs. By PCR 69%specimen HPV positive; 70% of skin lesions contained mucosal types HPV 6/11
190	94	120	44	H PCR	1,2,5,6,8,11, 16,18	79% in VVs, 42% in AKs, 33% in MBs, 43% in SCCs.
172	94	118	46	H PCR	1,2,3,5,7,10,37,41	60% in VVs, 53% in AKs, 25% in MBs, 55% in SCCs, 60% in BCCs. In 4 lesions double or triple HPV infection. In SCC HPV 41 and 29. 15% of lesions containing EV-specific types.
14	95	53	26	PCR	EVA-types	81% in SCCs.No HPV 5 and 8 in SCCs of RTRs detected. 30% > one HPV type.
43	95	96	44	PCR		93% in AKs, 40% in MBs, 57% in KAs, 80% in SCCs, 50% in BCCs. In 42 % mixture of HPV. In single step PCR HPV in 21% skin cancers, with nest. PCR 80% HPV in the same skin cancers. No HPV 5/8 or mucosal HPVs 6/11 detected.
173	96	25	19	PCR	41,29,69	65% in SCCs, 60% in BCCs. In ICIs 31% of SCCs and 36% of BCCs HPV+.
29	99		31	PCR	5	HPV 5 detected in 45% of plucked hairs from RTRs at one or more body sites (arms/legs /eyebrows).
44	00	176	78	PCR	EVA-types 5,8,9,12,14,15,19,20,21,22,23,24,25,29,36,37,38,47, and novel EVA-HPVs	0-50% in VVs, 48-50% in AKs, 60% in MBs, 73% in SCCs, 33% in BCCs.Prevalence of EVA-HPV DNA equally high in clinically benign keratotic skin lesions from RTRs with and without skin cancer history (55% and 53%). In both groups a large variety of EVA- HPV types was found: higher prevalence of EVA-HPV was found in sun exposed benign skin lesions from RTRs with a history of skin cancer.
13	00	351	?	PCR	EVA-HPVs 2,3,5,8,9,10, 12,14,15,17, 19,20,21,22, 23,24,25,28,29,36,37,38, 47,49,57	Almost all RTRs are infected persistently with one or more EVA- HPV types or other cutaneous HPV types. The frequency and distribution of these HPV types were similar in hyperkeratotic papillomas (77.5%), SCC (77.8%)and AK (67.9%). Their frequency was lower in BCC (35.7%) and normal skin (32.3%).
136	00	39	26	PCR		50% SCCs HPV + using degenerate primers, 69% SCCs HPV + with addition of nondegenerate primers
137	03		55 OTRs	PCR		75% in SCCs. HPV prevalence 75% in OTRs versus 47% in ICIs (p=0.2). Multiple HPV infections more in OTRs than ICIs, 52% versus 19%. Increasing HPV DNA frequency from normal skin (16%), premalignant lesions (39%) and CSCCs (69%). HPV 5 and 8 only in OTR and mostly in SCCs

Also in ICIs, HPV DNA is frequently detected, also with sometimes reported differences between studies with regard to HPV-types and frequencies found in skin tumors. For instance, Meyer et al reported prevalence of HPV DNA in 75% of SCCs of OTRs, and 47% of SCCs in ICIs. In warts and premalignant skin tumors comparable HPV prevalence were present in both patient populations[137]. HPV types 5 and 8 were more frequent in SCCs (26%) than in benign and premalignant lesions and all HPV 5 and 8 positive SCCs were from OTRs. Recently, positive serologic positive findings for HPV type 8 proved associated with SCC occurrence in ICIs, independently from other risk factors as sun-exposure and positive family history of skin cancer[126]. Comparable to Meyer et al, Rübben et al also reported the same distribution of HPV types in common warts from ICIs and immunocomprised patients [164]. Iftner et al. reported presence of HPV DNA in 90.9% of warts, 60.4% of premalignant lesions and 59.7% of SCCs in ICIs. High risk mucosal HPV types (e.g. HPV 16 and 31) were present in skin tumors of these ICIs and linked to NMSC [89]. Shamanin et al also found more frequently HPV DNA in SCCs of RTRs compared to ICIs and the spectrum of HPV types differed substantially. High risk mucosal types were only present in malignant skin tumors from RTRs, while in ICIs low risk mucosal types were found [173].

In conclusion, with the development of degenerate PCR methods a high prevalence and broad spectrum of HPV types has now been found in premalignant and malignant skin lesions from both immunosuppressed and immunocompetent individuals. The different spectrum of HPV types reported in malignant lesions from immunocomprised patients and RTRs and association of NMSC occurrence in ICIs with high risk HPV types, both suggest that viral infection acts as a cofactor in tumor development, along with solar radiation and other environmental exposures, although at present mechanistic data are lacking.

PART II

CURRENT KNOWLEDGE ON THE ROLE OF P53 AND INK4A-ARF (P16-P14) IN CUTANEOUS SQUAMOUS CELL CARCINOGENESIS

1.2.1
p53 and INK4A-ARF in the cell cycle control

Cell growth is controlled by two main pathways, one involving the retinoblastoma protein (pRb), regulating exit from the G1 phase of the cell cycle, and one involving the p53 protein that induces growth arrest or apoptosis in response to cellular stress. Crosstalk between the p53 and pRb pathways are provided by MDM2, by p21 and an additional important bridge is provided by the INK4a-ARF locus located on human chromosome 9p21 (see **Figure 2A**). This locus, by alternative transcripts encodes for two proteins $p16^{INK4a}$ (exons 1α, 2 and 3), and $p14^{ARF}$ (exons 1β and 2).

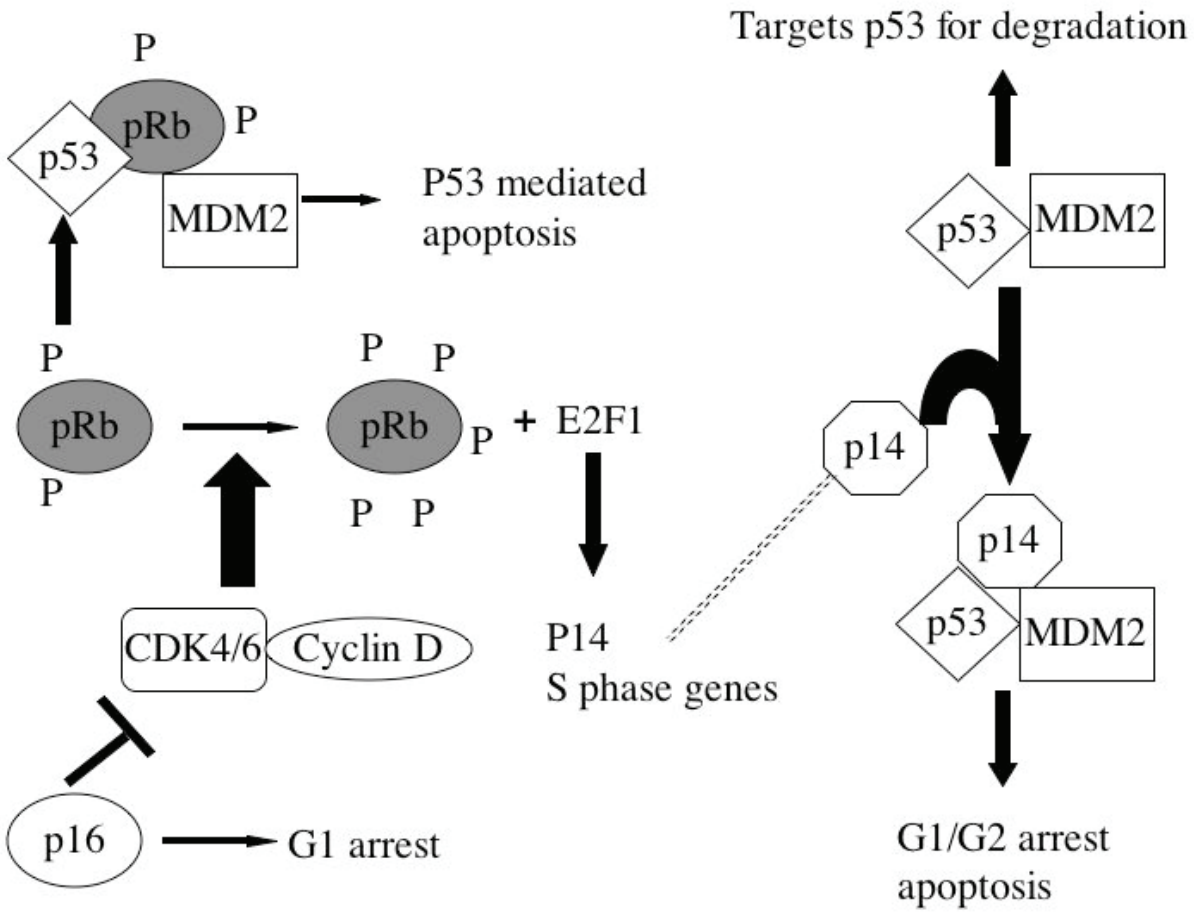

FIGURE 2A pRb is inactivated by phosphorylation, and inactivation of RB releases the E2F1 transcription factor from the RB complex. This factor regulates the expression of many genes involved in entry and progression through the S phase of the cell cycle. Overexpression of p16 inhibits entry and progression into the S phase of the cell cycle by inhibition of CDK4 and 6, thereby preventing phosphorylation of RB and release of E2F. Loss of p16 may allow excessive activity of CDKs, promoting RB phosphorylation and proliferation of tumor cells.
P14 interacts directly with MDM2 and sequesters MDM2 in the nucleolus and enables p53 stabilization. Also E2F1-induced activation of p53 is mediated by p14, which is transcriptionally activated by E2F1 and functions in p53 activation and stabilization by negating the effects of MDM2 on p53.

The p16-CDK4/6-RB pathway

P16 is a member of the INhibitor of cyclin-dependent Kinase 4 (INK4) family of proteins, which are capable of binding to and blocking the activity of cyclin dependent kinases (CDKs)4 and 6. CDK4/6 kinases associate with type D cyclins and these complexes are responsible for phosphorylation of pRb at G1 of the cell cycle. Sequential phosphorylation of pRb by CDK4/6 complexes followed by CDK2 complexes is thought to be critical for progression through G1 and entry into S-phase of the cell cycle. RB phosphorylation leads to release of E2F, which transactivates many genes important for mitosis.

By binding to CDK4/6, p16 can inhibit the phosphorylation of pRB with subsequent inhibition of E2F release and arrest of the cell cycle in G1 phase and suppression of cell proliferation. Functional or structural loss of p16, could therefore lead to cell cycle propagation of potential genetically damaged cells and subsequent risk of tumor development. Links between the p16 and p53 pathways are present and mediated by p14, which is formed by an alternative reading frame in the INK4a gene, which is shared by p16 and p14 [163]. Therefore, loss of the INK4a gene disrupts two cell control pathways, one through p16/CDK4/6/pRB and the other through p14/MDM2/p53.

Expression of p16 does not displace type D cyclins from already preformed cyclin D/CDK complexes, but type D cyclins have a very rapid turnover allowing p16 to out complete the cyclins for binding to CDK4/6. The p16/CDK complexes have a long half-life.

The expression of pRb is essential for transducing p16's signal to cause a cell cycle arrest. In absence of pRb, forced induction of p16 does not cause cell cycle arrest.

A negative feedback loop exists between pRb and p16. Transcription of p16 is repressed by pRb [91,163,174].

The p14-MDM2-p53 pathway

The human (***p14***) form of ARF is capable of inducing cell cycle arrest at the G2/M as well as at the G1/S phase[91,163,174]. Unlike p16, ARF does not bind to CDKs. ARF interacts with MDM2, an oncoprotein that negatively regulates p53 by (a) binding within the transcriptional domain of p53, inhibiting its transcriptional activity, and (b) acting as an E3 ubiquitin ligase, thereby promoting p53 proteolysis through the ubiquitin/proteasome pathway and it shifts MDM2 from the nucleus to the nucleolus. Transcription of MDM2 itself is controlled by p53 in response to cellular stress. Thus p53 and MDM2 form a loop creating a tight check over p53 protein level and function[54].

P14 interacts directly with MDM2 and sequesters MDM2 in the nucleolus and enables p53 stabilization.

Also E2F1-induced activation of p53 is mediated by p14, which is transcriptionally activated by E2F1 and functions in p53 activation and stabilization by negating the effects of MDM2 on p53 [54,174]. This responsiveness of p14 to E2F1 makes p14 an important nexus between the pRB and p53 pathways. The p14-E2F1 interplay enables the cell to sense oncogenic stimuli transduced through the RB-pathway, such as p16 inactivation or cyclin D overexpression. P14 is also induced by typical oncogenes such as myc and ras, and some viral oncogenes. Furthermore, a negative feedback loop exists between ARF and p53 [193]. Thus p14 plays a central role in cell cycle control, by integrating all these stimuli and by targeting at least two of the key pathways in control of cell cycle, namely the p53 and RB pathway.

P53 is a transcription factor that enhances the rate of transcription of several genes among which are p21 and MDM2. Normally in a cell the p53 protein level is low due to a relatively short half life (20 min). Several different types of DNA damage can activate p53 (for instance UV radiation), leading to a rapid increase in p53 level of the cell and activation of p53 as a transcription factor. The downstream events mediated by p53 activation take place by two major pathways : cell cycle arrest and apoptosis [116].

P53 activation turns on the transcription of its downstream genes, for instance p21. P21 binds to a number of cyclins and CDK-complexes, and binding of 2 molecules of p21 per complex inhibits kinase activity and blocks cell cycle progression. P21 also binds to PCNA and these complexes are thought to block the role of PCNA as a DNA polymerase processing factor in DNA replication. Thus p21 can act on cyclin-cdk complexes and PCNA to stop DNA replication (G1 arrest).
P53 plays a role in triggering apoptosis under several physiological conditions and p53 can also initiate apoptosis in response to the expression of a viral or cellular oncogene or the absence of a critical tumor suppressor gene product (Rb).
In conclusion, p53, p16, and p14, all act as proliferation and growth inhibitors in normal cell cycle control and therefore alteration in one of these (tumor suppressor) genes or proteins could lead to enhanced carcinogenesis. In addition, alteration in only one of these three tumor suppressors will have influences on both others, since p14 forms a nexus between the p16/pRB and p53/MDM2 pathways. In the present chapter the current knowledge on the role of these three tumor suppressors in cutaneous squamous cell carcinogenesis will be reviewed.

1.2.2
p53 in cutaneous squamous cell carcinogenesis of immunocompetent individuals and renal transplant recipients

1.2.2.1 TP53 mutations

In **table 2A** the frequencies of p53 mutations in ICIs and RTRs in CSCCs as previously reported in the literature are summarized. Reported frequencies in RTRs range from 8-43%, in ICIs from 18-50%. In most studies patient numbers are relatively small.
McGregor et al. detected mutations in p53 in 43% (9/21) of SCCs in RTRs and in 50% (3/6) of SCCs in ICIs. In 75% of the transplant tumors and 100% of the non transplant tumors p53 mutations were consistent with damage caused by UV radiation [129]. Mc Gregor concluded that both the type and frequency in p53 mutations, predominantly of an UV-related nature, was similar in transplant NMSC when compared to sporadic NMSC. Furthermore, in both groups they found that p53 mutations were present irrespective of HPV status. This latter finding is in contrast to anogenital cancer in which concurrent presence of high risk HPV and p53 mutation is uncommon, due to efficient binding and thereby functionally inactivating of p53 and RB by E6 and E7 proteins respectively, both encoded by the high risk HPV.
Bennett et al. and O'Connor et al. detected a much lower mutation frequency in SCCs of RTRs, with only 8% (2/25 cases) [10] and 11% (1/9 cases) p53 mutations respectively [144].
Soufir et al. reported p53 mutations in 5/21 (24%) of sporadic SCCs [187], in one case with associated p16 mutation.
Bolshakov et al. analyzed p53 mutations in aggressive SCCs with aggression being defined as tumor size above 2 cm, invasion in muscle, bone or cartilage or regional or distant metastasis. In aggressive SCCs 35% (28/80 cases) contained p53 mutations, while in non-aggressive SCCs 50% (28/56 cases) contained p53 mutations [23]. In both aggressive and nonaggressive SCCs most p53 mutations were C to T and CC to TT transitions at dipyrimidine sequences, suggesting induction by UV radiation (71% of all NMSCs studied contained UV-signature mutations). Whether tested SCCs were all sporadic or partly derived from special patient groups as for instance RTRs in this study is not mentioned.
Boldrini et al. found p53 mutations in 2/11 (18%) SCCs, probably sporadic tumors [22].

Table 2A. Reported p53 mutation frequencies in the literature in CSCCs in renal transplant recipients (RTRs) and immunocompetent individuals (ICIs)

Reference	RTR	ICI/sporadic	Exons p53
129	43%	50%	2-11
187		24%	4-9
10	8%		5-8
23		35-50% in aggressive vs. non-aggressive SCCs*	4-9
22		18% *	4-9
144	11%		5-8

*Most probably sporadic

In ***special patient groups, other than RTRs***, most studies report higher frequencies for p53 mutations compared to frequencies reported in CSCCs of immunocompetent individuals. In addition, tumors more often have multiple p53 mutations: for instance Stern et al. reported 61% p53 mutations in 51 CSCCs in PUVA treated (psoriasis) patients [191]. Of these mutations, 41% were PUVA type and 44% UV type. In Epidermodysplasia Verruciformis

(EV), 62.5% (5/8 cases) of CSCCs contained p53 mutations. Mutations were also present in 2 AKs (40%), 3 Bowen's disease (33.3%) and in 1 benign lesion. In 5/9 (55%) cases, mutations characterized by sequencing proved C->T transitions and were considered as UV-signature [147].
Soufir et al. found 44% (8/18 cases) p53 mutations in exons 4 through 9 of the p53 gene in Xeroderma Pigmentosum (XP) patients (contrasting to 24% in sporadic tumors); of these tumors 58% also had an INK4a-ARF mutation [187]. XP is an autosomal recessive disorder associated with a germ line nucleotide excision repair defect. This defect prevents removal of a wide array of DNA lesions, including those induced by UV radiation. This leads to a high frequency of sunlight induced skin cancers in these patients. The DNA repair defect, forms an explanation for the increase in mutational frequencies in p53 and also INK4A-ARF in skin cancers from these patients compared to sporadic skin cancers [187].

In conclusion, there are a limited number of studies on the incidence of p53 mutations in CSCCs in RTRs, which show a wide variation in the frequency of p53 mutations that might be at least partially attributable to differences in the number of p53 exons, tested. Frequencies seem lower or comparable to those in ICIs. UV-related mutations in p53 are common in both patient groups (range 71-100%).

1.2.2.2 p53 protein expression

Immunohistochemistry has been used to detect modified p53 protein, because many of the mutations in the p53 coding region result in a structurally altered, inactive protein that is more stable than its wild type counterpart, resulting in higher levels of protein detectable by antibody.

Findings on p53 expression in (pre) malignant squamoproliferative lesions in ***ICIs*** reported in the literature are listed in **Table 2B.** In all studies p53 staining was nuclear. As can be read from the listed studies variable results are reported, with p53 expression in AKs varying from 69-92%, in BDs from 9.1%-100%, and in SCCs from 50% to 100%. But as also can be read from the table different antibodies and dilutions have been used and varying quantitative scoring systems.

P53 staining in normal skin was reported as either absent [90,128,214], or as having low detectable expression throughout the epidermis in sun-exposed skin [214].

Table 2B. Reported frequency of p53 protein expression in (pre) malignant squamoproliferative lesions in the general population.

Reference	P53 antibody	AK	MB	SCC
113	Pab1801, (Oncogene Science, Cambridge, MA		9.1% >10% p53 +	
214	DO-7, DAKO		80% moderate to strong +	100% moderate to strong +
120	DO-7, Novocastra, UK	79.2% moderate to strong +	100% +	38% >50% tumor cells p53 +
90	BP53-12, Japan Tanner, Kobo, Japan 1:20			50% moderate to strong +
157	DO-7, DAKO, Denmark 1: 200	Up to 69% +	55.6% +	69.1% +
183	CM1 1:1000-1:8000		57.7%+	
177	DO-7, DAKO Japan 1:100			75% +
146	DO-7, Novocastra, England 1:100	92% +	71% +	55% +
128	DO7, Novocastra, UK 1:50			94% +

Literature is sparser on p53 expression in tumors of ***RTRs.***

Ferrandiz described more prevalent accumulation of p53 protein (used antibody DO-7, Novocastra, UK) in AKs and SCCs of RTRs, percentages of 82% and 85% respectively, when compared to equivalent lesions in ICIs with 40% p53 positivity in both actinic keratoses and SCCs [66].

McGregor et al. found no difference in the overall prevalence of p53 staining in transplant and non-transplant associated skin tumors: 43% p53 immunostaining in SCCs of RTRs and 55% of SCCs in the general population. 57% of verrucous keratoses in RTRs and 62% of AKs in the general population were p53 positive. In this study none of the lesions contained oncogenic HPV (PCRs for HPV types 16,18,31,33,5 and 8 were performed), suggesting that p53 mutation rather than p53 inactivation by HPV-encoded E6 oncoprotein plays a role in carcinogenesis of both patient groups [130].

O'Connor et al. found p53 expression in 70% (7/10) of SCCs of RTRs, compared to 40% (2/5 cases) p53 expression in ICIs. Only one of all p53 positive SCCs contained a p53

mutation. The number of positive tumor cells varied from >30% to 80%. In 8 of all 15 cases HPV was present (EV-HPV types), in 6 RTRs and 2 ICIs, not related to p53 expression. Viral warts from both ICIs and RTRs contained p53 in 5/15 cases, and in most cases staining was weak (<10%) in a few isolated cells, located basal or suprabasal [144].

In conclusion, p53 expression in (pre) malignant skin lesions of RTRs is reported frequently (range 43-70%) with most studies reporting less prevalent p53 overexpression in equivalent lesions in ICIs. P53 overexpression is not synonymous with p53 mutations, and in skin lesions thus far no correlation of p53 expression with presence of oncogenic HPVcould be demonstrated.

1.2.3

INK4A-ARF in cutaneous squamous cell carcinogenesis of immunocompetent individuals and renal transplant recipients

Inactivation of p16 and p14 can occur by a variety of genetic mechanisms including mutations and deletions. In addition to genetic mechanisms, hypermethylation of the CpG islands of the separate p16 and p14 promotors, is a significant means of transcriptional silencing [143,163,182]. There is now compelling evidence that DNA methylation errors in cancer cells can alter the expression of critical genes and contribute to carcinogenesis [163]. In chapter 2.3.1 an update on INK4A-ARF mutations in cutaneous carcinogenesis will be given, and only briefly mention other genetic events concerning INK4A-ARF, since these were not further studied in the present thesis.

1.2.3.1 INKA-ARF mutations

So far there is a restricted number of studies on INK4A-ARF mutations in skin carcinomas: see **Table 2C.**

Reported frequencies of p16 mutations in ***sporadic CSCCs*** varies from 0% (0/6 cases) [35] to 20% (resp. 4/20 CSCCs [188] and 8/42 cases of CSCCs [187]). P14 mutations in the sporadic CSCCs have a reported frequency of 17% (7/42 cases) [187].

Chang et al. found no p16 mutations in 6 SCCs, including 2 with metastases. Only exons 1 and 2 of p16 were amplified [35].

Kubo et al. found INK4A-ARF mutations in 3/21 (14%) sporadic CSCCs, all in exon 2 affecting both p16 and p14. The two SCCs arising from sun-exposed skin, contained a CC->T mutation at a dipyrimidine site and a deletion of 21 base pairs. Both types of mutations were not specific and uncommon to UV mutagenesis. The third mutation was a C->T transition, occurring at a dipyrimidine site, but this SCC originated in a scar in a non-sun exposed site [110].

Soufir reported 66% UV-type mutations in sporadic CSCCs (two tandem CC:GC to TT:AA transitions and two C:G to T:A transitions at dipyrimidic sites) [188], with only 5% (1/21 cases) of sporadic SCCs having combined p16 and p53 mutation [187]. In addition methylation analysis of p16 was performed, and was present in 8/20 SCCs (40%), three of which harboring a p16 mutation[188].

At the moment only one previous study was concerned with INK4A-ARF mutations in ***iatrogenically immunosuppressed patients***, which were not further specified [32]. A total of 40 SCCs was studied, 30 samples from immunosuppressed patients and 10 from ICIs. In 4/40 (10%) SCCs, INK4A-ARF mutations were present. All mutations were C to T nucleotide changes or double substitutions CC to TT, consistent with UVB fingerprint type of mutation (100% UV type). In ICIs 3/10 SCCs contained mutations, 2 combined p16/p14 mutations, 1 mutation affected only p16. In immunosuppressed only 1/30 SCCs contained a combined P16/p14 mutation. In this study, LOH for 9p21 was found in 13/40 (32.5%) of the tumors. No samples showed microsatellite instability for this locus. Methylation of the p16 promotor was found in 13/36 (36%) of cases, for the p14 promotor in 16/38 (42%) cases, and 4 samples were methylated for both the p16 and p14 promotor.

So in this study genetic/epigenetic events (mutation, methylation, immunohistochemical expression) for p16 and p14 were found to be more common in ICIs compared to immunosuppressed patients. An overall frequency of 76% 9p21 alterations was found for

both p16 and p14, with promotor methylation being the most common mechanism of gene inactivation.
Studies on INK4A-ARF mutations in ***RTRs*** so far have not been performed.
In ***special patient groups, other than RTRs***, reported frequencies for INK4A-ARF mutations in CSCCs are higher than those reported in the general population.
In SCCs form PUVA-treated psoriasis patients, 42% (11/26) SCCs contained 19 INK4a-ARF mutations: 10 mutations affected p16, 6 affected p14, and 3 affected both. UV-type mutations were present in 58%, three (16%) were ultraviolet and/or PUVA type [109].
In Xeroderma Pigmentosum (XP) patients, p16 and p14 mutations both have a reported frequency of 33% (6/18 cases); all tumors in these patients with mutations in p16 also had mutations in p14 [187]. Furthermore tumors in XP patients often contained multiple mutations in p14, p16 and p53 and tumors with INK4a-ARF mutations almost all also had one or more mutations in the p53 gene (7/8, 88%).
54% of INK4a-ARF mutations in XP tumors (basal cell carcinomas included) were of UV-signature. Statistically positive associations were present between the frequency in p53 and p16 and between the frequency in p53 and p14.

Table 2C. Studies on INK4A-ARF mutations in SCCs

Ref	Freq. p16 mutations	Freq. p14 mutations	Patient characteristics	Analyzed exons of INK4a-ARF
35	0% (0/6)	Not done	Sporadic SCCs	1,2
110	14% (3/21)	14% (3/21)	Sporadic SCCs	1,2,3
188	20% (4/20)		Sporadic SCCs	2,3
187	19% (8/42)	17% (7/42)	Sporadic SCCs	1α, 1β, 2
32	30% (3/10)	20% (2/10)	Sporadic SCCs	1α, 1β, 2
	3% (1/30)	3% (1/30)	Immunosuppressed patients	
109	50% (13/26)	35% (9/26)	SCCs from PUVA treated psoriasis patients	1α, 1β, 2, 3
187	33% (6/18)	33% (6/18)	SCCs from XP patients	1α, 1β, 2

In conclusion, so far the frequency of INK4a-ARF mutations in RTRs is not studied. In sporadic CSCCs INK4a-ARF mutations are reported in up to 30% of cases with often-combined p16 and p14 mutations. In immunosuppressed patients less epigenetic and genetic events in INK4a-ARF are described when compared to the general population with promotor methylation of p14 and p16 being the most frequent mechanism of gene inactivation (42%).

1.2.3.2 p16 protein expression

So far only a few studies have evaluated p16 immunoexpression in SCCs and precursors, mostly in ICIs. Hodges et al. recently described p16 positivity in all 10 (100%) probably sporadic AKs, SCC in situ lesions, and SCCs studied. They used the monoclonal p16 antibody G175-405 (Pharmingen, San Diego, CA (dil.1:25) and a semi quantitative scoring system. Normal skin was negative. AKs stained positive in the lower one third or lower one half of the epidermis. Transepidermal staining was only present in SCC in situ. 100% of the SCCs exhibited moderate to strong staining of the invasive component [87].

In contrast, Mortier et al. [140] found p16 expression in 66% of AKs (12 AKs studied) and only 10% of SCCs (10 SCCs studied), probably sporadic tumors, without specification of the used scoring method. The lower number of p16 positive SCCs in this study correlated with an increase of LOH at the p16 coding region 9p21 from 21% (3/14) AKs to 46% (17/37) SCCs, although for immunohistochemistry and LOH different samples were used. In contrast, they also describe p16 expression in normal skin with the use of the same antibody as Hodges et al. (G175-405, Pharmingen).

Combined mutation analysis with immunohistochemistry for p16, so far was only performed by Chang [35] et al. though on a very small number of probably sporadic SCCs (6 cases): 2/6 SCCs showed positive nuclear staining for p16 (anti-p16 antibody, C-20, Santa Cruz Biotechnology), and 4/6 SCCs were negative. In all cases wild type p16 was present and no mutations could be demonstrated, implicating that immunohistochemical findings cannot directly be correlated to underlying molecular alterations.

Nindl et al. studied p16 expression in relation to presence of HPV in normal skin (3), psoriasis (2), verrucae vulgares (VVs) (2), AKs (5), SCCIS 4), and SCCs (7) with the use of the monoclonal antibody E6H4 (MTM laboratories, Heidelberg, Germany; dil. 1:200) and a semi quantitative scoring system. P16 expression was absent in normal skin, psoriasis, and VVs. All AKs, SCCISs and SCCs showed p16 expression, with the highest percentage of positive lesional cells in SCCIS (>70%) and in all AKs, SCCIS and SCCs either EV-associated HPV types (20,24,5,14,15,17,25,8) or mucosal HPV types (16,6) were detected.

At present only Brown et al. [32] studied p16 expression in SCCs of both ICIs and immunosuppressed patients, which were not further specified. The monoclonal antibody p16INK4A/MTS1 Ab-7 (16PO7) (Neomakers, Fremont, California, dil. 1:100) was used, and only nuclear staining was considered positive. A semi quantitative scoring system was used. P16 staining was present in 20/40 (50%) of SCCs, of which 75% showed less than 25% positive tumor cells. In Immunosuppressed 16/30 cases (53%) were p16 positive, in ICIs 4/10 (40%). In 64% of cases there was concordance between the presence/absence of p16 protein expression and genetic results (mutation, methylation). In 70% of tumors with biallelic events, p16 protein expression was absent.

Summarizing, results with respect to p16 staining in SCCs of skin and precursors are conflicting and seem partly related to usage of different p16 antibodies, often-small patient numbers, and different immunohistochemical scoring methods. At this moment no studies regarding p16 protein expression in epidermal neoplasia of RTRs are available. Further studies with larger sample size are needed to clarify the relationship between p16 overexpression, etiological factors (HPV, sun exposition) and NMSC.

1.2.3.3 p14 protein expression

At this moment only 1 study has been performed regarding p14 protein expression in (pre) malignant squamoproliferative skin lesions. Brown et al. [32] studied p14 expression in 40 SCCs, 30 from immunosuppressed patients and 10 from immunocompetent persons. They used a goat polyclonal antibody p14 ARF (C-18)(sc-8613) (Santa Cruz Biotechnology, Inc.) in a 1:100 dilution, a semi quantitative scoring method and considered only nuclear staining as positive.

P14 expression was seen in 17/40 (43%) of cases: 12/30 (40%) p14 positive samples in immunosuppressed, and 5/10 (50%) p14 positive SCCs in ICIs. In 62% there was concordance between the presence/absence of protein expression and genetic events. In all tumors with biallelic events, p14 expression was absent.

So far studies on p14 expression in premalignant squamoproliferative lesions of specifically RTRs are lacking.

PART III

SYSTEMIC RETINOID TREATMENT IN SKIN CANCER

1.3.1
Retinoid effects on the epidermis

Retinoids are a class of compounds that includes the natural and synthetic analogues of retinol or vitamin A. Vitamin A has long been known for its importance in promoting general growth, in regulating proliferation and differentiation of epithelial tissues, and in maintaining visual function and reproduction [170].
Retinoids have a broad range of effects on the epidermis, and the most important and relevant to the studies described in this thesis will be mentioned here. First, I will briefly mention normal keratinisation in the epidermis and in benign and (pre) malignant epidermal neoplasia since retinoids have a profound effect on epidermal keratinisation and the effect on keratinisation of retinoids in epidermal tumors was one of the study objectives in this thesis.

1.3.1.1 Keratins in normal epidermis

Epithelial keratins comprise a heterogeneous group of acidic (type I) and neutral-to-basic (type II) proteins. As a general rule, they are coexpressed in specific pairings, each pair consisting of a type I keratin and II. For instance, in the normal adult skin, keratin pairs K5/K14 (46 and 58-kD resp.) predominate in the basal layer and larger keratins K1/K10 (67 kD and 56.5 kD resp.) in the suprabasal compartment [28,185].
The type I keratins K13 and K19 (52 kD and 40kD resp), are usually expressed separately in adult epithelia. Combined expression occurs only in fetal skin (see **Figure 3A**). Both keratins are absent in normal skin of adults except for certain body sites such as penile foreskin that still contains K13. Furthermore, K13 in adults is abundantly present in internal stratified epithelia (for instance the esophagus) and associated with terminal differentiation or suprabasal expression [114,139,185,200]. K19 expression in adults is found in simple epithelia, such as most glandular epithelia.

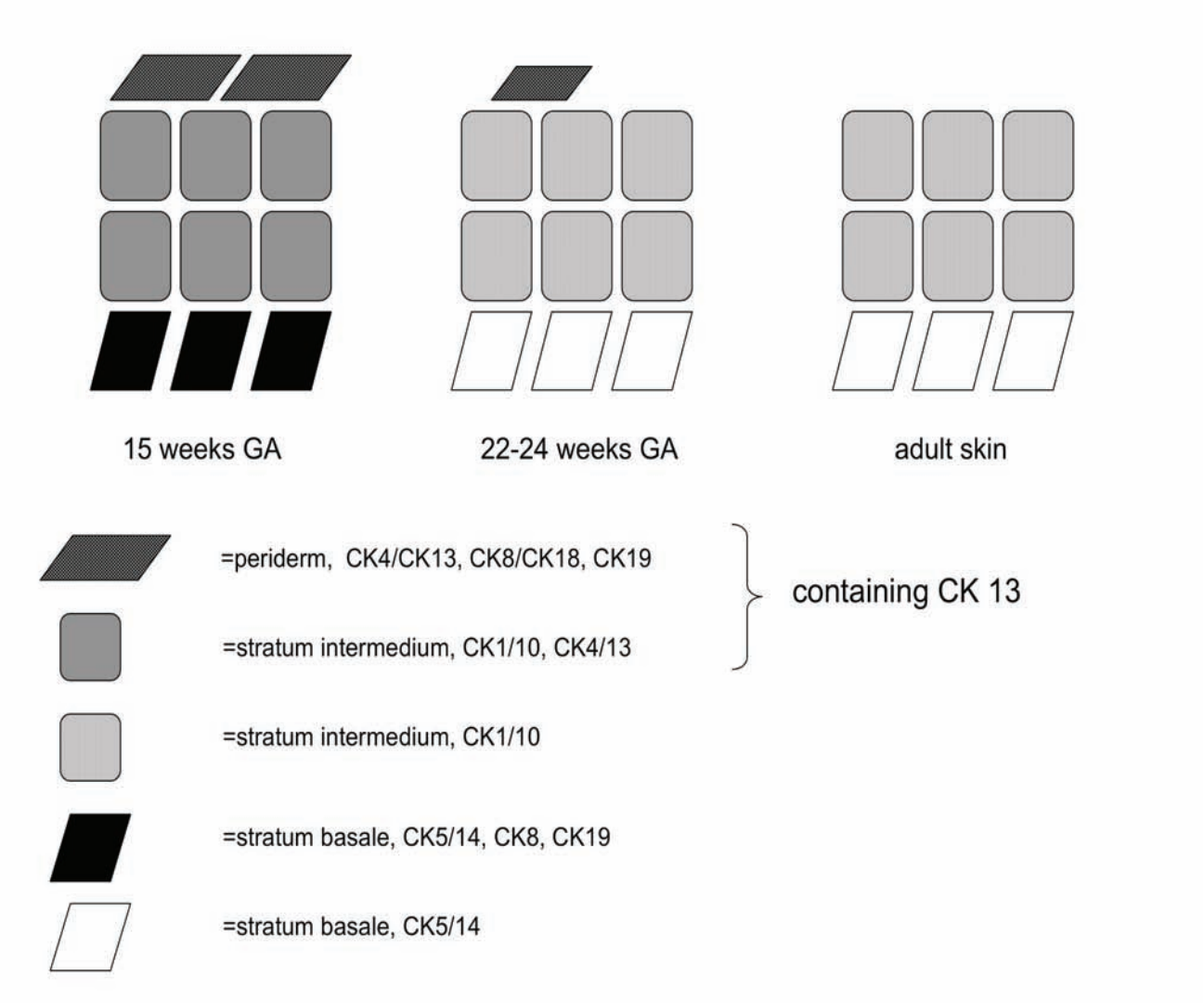

Figure 3A Expression of the different keratins during the development of the epidermis. Keratin expression at 15 weeks of gestational age (GA), at 22-24 weeks of GA, and in adult skin are depicted. Keratin 13 (K13) is only present in fetal skin.

1.3.1.2 Keratinisation in warts, premalignant and malignant skin tumors

Proby et al. assessed the profile of keratin expression in benign warts, dysplastic skin lesions and SCCs in RTRs and compared expression patterns with identical lesions in immunocompetent controls (ICIs)[153].
In warts, besides also normally present suprabasal keratins K1 and K10, immunoreactivity for hyperproliferative keratins K6 and K16 was seen usually throughout the suprabasal compartment. Suprabasal K17 expression was found. Simple epithelial K7, K8, and K18 were not expressed in warts. Findings were similar in warts of RTRs and ICIs.
Dysplastic lesions frequently showed marked delay in suprabasal immunoreactivity for K1/10 with marked expansion of basal cell marker LH8. In addition, a combination of basal and suprabasal K17 expression was seen. Simple keratins K7 and 8/18 were not expressed in dysplastic lesions.

The profile of keratin expression of SCCs from RTRs did not differ from SCCs of ICIs. Expression of K1/10 was lost. In SCCs extensive K17 positivity was seen with panepithelial staining throughout areas of invasive tumor. K8/18 was only detected in the least differentiated SCCs, as was K19.
K13 was negative in all lesions.
Malecha and Kuruc also reported absent K13 expression in SCCs of the skin [114,122].
Markey et al. and Ichikawa et al. found comparable keratin expression in epidermal dysplasia and in situ carcinomas as described above [88,123]. Both reported in situ lesions a few K7/K8/K18 and K19 positive cells. In well differentiated SCCs K5 and 14 staining was preserved, while poorly differentiated ones showed widespread loss of both K14 and K5 and loss of other basal markers. All SCCs showed K1/10 loss, and no alternative terminal differentiation such as oral/esophageal (K4/13) or corneal (K3/12) keratin expression could be demonstrated. In all SCCs widespread expression of keratins normally present in simple epithelia, like K7/K8/K18 and K19, was present [123].
In conclusion, cutaneous malignant transformation is heralded by a switch from production of larger MW keratins normally present in the epidermis (K1/10) to smaller MW keratins (K8, 18 and 19) characteristic of fetal skin and simple epithelia and that the extent of shift correlates with the histological grade of the tumor as already stated by Smack et al. [185]. K13 expression is reported to be absent in (pre) malignant epidermal tumors.

1.3.1.3 Effects of retinoids on epidermal keratinisation, differentiation, and proliferation

With respect to keratinisation and differentiation, retinoids influence expression of cytokeratins. In skin, retinoids induce keratins that are normally not present in normal adult human skin, but are present only in embryonic skin. Data from animal and in vitro studies with cultured human keratinocytes have indicated that retinoids repressed expression of epidermal differentiation-specific keratins (K1/K10) and strikingly reinduced expression of two lower molecular weight keratins, corresponding to K13 and K19 respectively [55,106,107,202]. Since these two keratins are coexpressed in fetal skin [139,200], this type of retinoid-induced differentiation is also termed embryonic differentiation. Furthermore retinoids in vitro increase the keratins K4, K15, and K6/K16 in keratinocyte cell cultures [185]. Keratinocytes in vivo, when exposed to topical retinoids in healthy volunteers, display an increase in K13 and K6/16 synthesis, but no alterations in K1/10 and K14 expression [161]. Interestingly, in contrast to normal keratinocytes, in psoriatic skin systemic retinoid treatment seems to reverse the enhanced K6/16 in psoriasis, with restoration of suprabasal K1/10 expression. This illustrates that retinoid response of keratinocytes in vivo and in vitro do not always correlate and that normal keratinocytes respond differently to retinoid than diseased keratinocytes [119,185].
Epidermal proliferation is also influenced by retinoids. Some have reported that topical retinoids increase the uptake of tritiated thymidine in human epidermis and pilosebaceus follicles, while others noted decreased numbers of mitotic figures after oral administration of vitamin A, suggesting that local irritation may explain the increased labeling after topical application of retinoids as reviewed by Elias [57]. Furthermore oral retinoids were reported to produce acanthosis and increased epidermal labeling index in mice, although this proved only a temporally effect with reversion to normal after 2 weeks. Light microscopy after topical and systemic retinoid therapy also demonstrated only temporally acanthosis or psoriaform hyperplasia [57]. Hyperplasia of epidermis after retinoid stimulation is also accompanied by an increased shedding of corneocytes. This shedding is probably at least partially attributable to dyshesion, which correlates with the loss of desmosomes and the formation of intracellular

and extracellular amorphous materials in the upper dermis. Ultrastructural studies demonstrated that retinoids reduced the size of desmosomes and increased the number of gap-junctions, reduced the density of tonofilaments and induce the formation of gaps in the epidermal basement membrane [56,57,197].

Retinoids are thought to induce growth or inhibit growth dependent on the cell type and state of the studied tissue. With regard to cell type, for instance the sensitivity of various type of cultured keratinocytes to retinoids appears to reflect the degree of cornification of the tissue of origin in vivo: conjunctival keratinocytes proved more sensitive to retinoid than epidermal keratinocytes, probably due to either divergence of differentiation program in these keratinizing epithelia and/or different sensitivity to vitamin A.

Regarding cell state, in normal epidermis retinoids are reported to induce DNA synthesis and increase proliferation with increased desquamation. This contrasts with growth inhibition by retinoids in hyperproliferative states, for instance psoriatic skin [197].

These pluripotent effects of retinoids have made them an established treatment for various skin disorders such as psoriasis, acne, and keratinisation disorders [170]. In addition, retinoid treatment has been employed in the prevention and treatment of skin ageing and skin cancer [46,118], the latter being the subject of investigation in the present thesis.

1.3.2
Retinoid receptors in skin

The ability of retinoids to modulate gene expression is the most plausible mechanism by which they can modulate differentiation and growth of malignant cells or suppress the progression of premalignant cells to frank neoplastic lesions by redirecting their differentiation [119].

To modulate gene expression, retinoids must transmit signals to the nucleus. Both cytoplasmic and nuclear retinoid binding proteins have been identified. Cytoplasmic retinoid binding proteins, like cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP), have been identified in human skin and (un) differentiated cultured keratinocytes. These proteins probably function in transport of retinoids to the cell nucleus.

Nuclear retinoic acid receptors are members of a large family of nuclear receptors for steroids, vitamin D, and thyroid hormones. There are three Retinoic-Acid-Receptor (RAR) subtypes (RAR-α,β and γ) and three Retinoid-X-Receptor (RXR) subtypes (RXR-α,β and γ) and each subtype has different tissue distribution.

Human epidermis expresses RAR-α, RAR-γ, RXR-α and RXR-β. Total RXR protein levels are fivefold greater than RAR levels. RXR-α is the skin's predominant RXR, accounting for 90% of the RXR protein in skin, while RAR-γ is the most predominant RAR accounting for 87% of RAR protein in skin [67,119,215]. The RXRs form heterodimers with RARs, and therefore it is highly likely that the RAR-γ/RXR-α heterodimer is the principle transducer of the retinoid signal in human skin.

Different retinoids have different receptor specificity. At present there are three generations of synthetic retinoids [170]. The first generation is the group of non-aromatic retinoids. One of these, all-trans retinoic acid (ATRA), is used topically for a variety of dermatologic conditions and also in prevention of skin cancer. Of the second generation of mono-aromatic retinoids, the compounds etretinate and acitretin, both have been employed in systemic prevention and treatment of skin (pre)cancers. ATRA especially binds to RAR receptor subtypes. Acitretin does not bind, but activates RAR receptors. Bexarotene is an example of the third generation of polyaromatic retinoids or arotinoids, and binds especially to RXR receptors[47].

During squamous cell carcinogenesis decrease in nuclear retinoid receptors is reported : RXR-α was relatively more decreased in AKs, and RAR-γ relatively more in SCCs, suggesting that suppression of RXR-α may be an earlier event in skin carcinogenesis than RAR-γ suppression [215]

1.3.3

Oral/systemic retinoid treatment in prevention and treatment of skin (pre)cancer in RTRs

Several studies have suggested a beneficial effect of systemic retinoid treatment in RTRs and other high-risk patients with skin cancer [27,46,73,132,180,203] (**Table 3A**). Most of these studies are open studies in small groups of patients with different follow-up periods and variable treatment regimens. Most studies suggest a beneficial effect of systemic retinoid treatment with either etretinate or acitretin on the development of new skin (pre)cancers, but after discontinuation of the treatment report recurrent tumors [46,180,203]. Because retinoids only seem to work temporarily, long-term treatment seems indicated. Long-term retinoid therapy however has been associated with side-effects [47,118,196]. Therefore it seems advisable to restrict its use to RTRs actively developing large numbers of skin cancers [46].

Table 3A. Studies on systemic retinoid treatment in RTRs

Ref	retinoid	dose	duration	Patient no.	Effect	Type
180	etretinate	1mg/kg/day	6 mo	6	Resolution in 4/6 patients. Recurrent tumors in 2/6 after 6 months.	Open
132	acitretin	0.3mg/kg/day	5 yr	16	Significant reduction in tumors excised in 12/16 patients. Two patients lost due to side effects.	Open
27	acitretin	30 mg/day	6 mo	44	Reduction of SCCs and keratotic lesions in treated patients.	Double blind, placebo-controlled
160	Etretinate & topical tretinoin 0.025%	10 mg/day	6 mo	11	After 6 mo 50% decrease lesions in 6/8 patients, including 2/3 patients on tretinoin alone	Open
203	acitretin	0.5 mg/kg/day	15 mo	1	No new dysplastic lesions during treatment and disappearance of existing verrucosities. Return verrucous lesions 4 mo after discontinuation	Single case
73	etretinate	0.3 mg/kg/day	17 mo	11	Significant reduction of skin cancers compared to pretreatment. Improvement in hyperkeratotic lesions and in viral warts	Open
102	etretinate	50mg/day	8-13 mo	4	During treatment considerable reduction in number of SCCs. Rapid and accelerated tumor formation after treatment cessation in 2 patients	open

So far, only one study was randomized, double blind, placebo-controlled. It concerned RTRs with more than 10 keratotic skin lesions, in which 6 months treatment with acitretin 30mg/day lead to a significant reduction of new skin cancers in the treated group. There was

also a relative decrease in keratotic skin lesions in this group. Patients with a prior history of skin cancer benefited most [27]. McKenna et al demonstrated that patients with five or more tumors prior to treatment, benefited most from acitretin treatment[132].
Kelly et al. [102] found marked reduction in SCC incidence in 4 RTRs treated with oral etretinate (50mg/d); these patients developed 23 SCC in the 12 months before treatment, 6 SCCs on etretinate, and 34 lesions in the 12 months post-treatment. In 1 patient a rapid return and accelerated tumor development was noted with 21 SCCs in the 12 months after treatment cessation. So there was a rapid loss of chemopreventive benefit after discontinuation of retinoid treatment, which implicates that long-term treatment is necessary.

In none of these studies concerning systemic retinoid in RTRs, histology of (lesional) skin was evaluated. Furthermore no immunohistochemical markers for keratinisation, proliferation, or apoptosis were applied on skin biopsies during oral retinoid treatment in RTRs. Therefore in what way systemic retinoids act chemopreventive in skin (pre)cancer still needs further elucidation.
Regarding *topical* retinoid treatment with tretinoin cream (all-trans-retinoic acid) for photodamaged skin and actinic keratoses in ICIs, there are studies including histological and immunohistochemical changes in skin biopsies during treatment. Most studies concerning topical retinoids in photoaging report an increased epidermal thickness, increased granular cell layer thickness, decreased melanin content and stratum corneum compaction. Furthermore a bluish mucin-like material was noted in the stratum corneum[75,78,145,207,211,213].
Diminished or disappearance of dysplasia and atypia was found [78,105,213]. Some reported no clear dermal changes like for instance severity of inflammation or elastin and collagen content with light microscopy[75], while others found reduced elastosis, increased thickness of papillary dermis and decreased perivascular inflammation [15].
Immunohistochemistry was only performed in one study in which topical retinoic acid treatment in normal volunteers showed an induction of K6 and K13 expression, while K1, K10 and K14 retained a normal expression pattern [161].

PART IV

Outline of the thesis

Epidemiologic and etiological studies support an important role for UV radiation, immunosuppression, and HPV in squamous cell carcinogenesis in RTRs. There is a need for improved insight in the enhanced and more aggressive squamous cell carcinogenesis in renal transplant recipients. The role of the different risk factors in the enhanced carcinogenesis in RTRs needs further elucidation. In addition mechanistic data on the role of HPV in skin cancer development are currently lacking, although currently available data suggest its role is different from the role in cervical cancers. The influence of UV and HPV can expected to be reflected in immunohistochemical profiles for important tumor suppressors like p53 and INK4a-ARF. At this moment no studies regarding p16 and p14 protein expression in epidermal neoplasia of RTRs are available.
In RTRs, CSCCs tend to behave more aggressive with more frequent metastases. At the moment studies on p53 and INK4a-ARF mutations in metastatic skin tumors are not available. Since skin cancers are often multiple in RTRs, correct identification of the primary tumor responsible for metastasis is important in order to get more insight in what tumors behave aggressive in this patient group.
Systemic retinoid treatment is currently applied in RTRs actively developing skin cancers, but so far no studies have been performed to study effects of this treatment on markers for keratinisation, proliferation and apoptosis in skin in order to get insight in the way retinoids act chemopreventive.

Regarding the above, we developed the following research aims:
1. Is the influence of risk factors for cutaneous carcinogenesis (sun exposure, HPV, and immune status) different in RTRs compared to ICIs?
2. Can molecular tools aid in identifying the correct primary cutaneous squamous cell carcinoma in case of metastases in patients with multiple primary skin cancers?
3. How exert retinoids their chemopreventive effect in skin tumor development?

In order to address these research aims, the following research designs were formulated in this thesis:
1. Immunohistochemical studies for p53, p16, and p14 were performed in order to compare profiles for these markers in epidermal tumors from RTRs and ICIs, in order to get insight in the respective roles of the different etiological factors (HPV, sun exposition, immune status) in cutaneous carcinogenesis of both patient groups. By comparing profiles with data in the literature on profiles in HPV induced cervical neoplasia the role of HPV in skin cancer development of these patients might become clearer. In addition PCR for mucosal HPV types was performed and related to the expression of these markers.
2. Molecular study regarding the usefulness of p53 and INK4a-ARF mutation analysis in identifying primary CSCCs in case of metastases and multiple primaries was performed.
3. To elucidate the mechanisms of action of retinoids in (pre) malignant skin disorders in RTRs, immunohistochemical studies with markers for keratinisation and expression of cell-cycle associated proteins were performed in RTRs during retinoid treatment and after cessation of this therapy.

Chapter 2

TUMOR SUPPRESSORS P53, P16, AND P14 IN CUTANEOUS CARCINOGENESIS

This chapter was based on the following publications:

P16 and p53 Expression in (Pre) Malignant Epidermal Tumors of Renal Transplant Recipients and Immunocompetent Individuals

Willeke A.M. Blokx, M.D[1], Elke M.G.J. de Jong, M.D., Ph.D.,[2]Peter C.M. de Wilde, D.M.D., Ph.D.,[1]Johan Bulten, M.D., Ph.D.,[1] Monique M.G.M. Link,[1] Dirk J. Ruiter, M.D., Ph.D.,[1] Peter C.M. van de Kerkhof, M.D., Ph.D.[2]

Departments of Pathology[1] and Dermatology[2], University Medical Center St. Radboud, Nijmegen, the Netherlands

Mod Pathology 2003;16(9):869-878

P14 Expression and HPV in keratinocytic intraepidermal neoplasia (KIN) and cutaneous squamous cell carcinoma

Willeke A.M. Blokx, M.D.[1], Peter C.M. de Wilde, D.M.D., Ph.D.[1], Elke M.G.J. de Jong, M.D., Ph.D.[2], Sabine A.A.P. Aalders[1], Peter C.M. van de Kerkhof, M.D., Ph.D.[2], Dirk J.Ruiter, M.D., Ph.D.[1,], W.Melchers, M.D, Ph.D.[3]

Departments of Pathology[1,] Dermatology[2], and Microbiology[3], University Medical Center St. Radboud, Nijmegen, the Netherlands

Submitted

INK4A-ARF and P53 mutations in metastatic cutaneous squamous cell carcinoma

Case report and archival study on the use of Ink4a-ARF and p53 mutation analysis in identification of the corresponding primary tumor

Willeke A.M. Blokx, M.D[1]. Dirk J. Ruiter, M.D., Ph.D.[1], Marian A.J. Verdijk[1], Peter C.M. de Wilde, D.M.D., Ph.D.[1,] Riki W.Willems[1], Elke M.G.J. de Jong, M.D., Ph.D.[2]

Marjolijn J.L. Ligtenberg, Ph.D.[1,3]

Departments of Pathology[1,] Dermatology[2], and Human Genetics, University Medical Center Nijmegen, Nijmegen, the Netherlands[3]

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2.1

P16 and p53 Expression in (Pre)Malignant Epidermal Tumors of Renal Transplant Recipients and Immunocompetent Individuals

Abstract

Ultraviolet (UV) radiation is a prevailing factor implicated in the etiology of keratinocytic intraepidermal neoplasia (KIN) and squamous cell carcinomas (SCCs), as evidenced by the high frequency of UV-related mutations in the p53 and p16 tumor suppressor genes. In renal transplant recipients (RTRs), immunosuppression is considered another important risk factor in the enhanced carcinogenesis in these patients. So far, effects of UV and immune status on p53 and p16 immunoexpression in SCCs and precursors have not been studied.

The aims of this study were to assess (1) the relation between risk factors for carcinogenesis, sun-exposure, and immune-status, and p16 or p53 expression, and (2) to assess differences in p16 and p53 expression between KINs and SCCs.

Immunostaining for p16 and p53 was performed on paraffin-embedded sections of 23 low-grade KIN (LKIN) lesions, 28 high-grade KINs (HKINs) and 35 SCCs from 44 RTRs and 42 immunocompetent controls (ICIs).

In 74/86 lesions (86%) p53 was expressed and in 63/86(76%) lesions p16 expression was present. Negativity for both p16 and p53 was found in 4/86 (5%) cases, while combined p53/p16 staining was most prevalent (55/86 lesions, 64%). P16 staining proved independent of p53 expression (p=0.8), and immune status, sun exposure and histological diagnosis (LKIN-HKIN-SCC) had no influence on this independency.

Transplantation was associated with p53 expression in SCCs (p=0.02; power = 34%) caused by higher prevalence of p53 negative SCCs in RTRs than in ICIs (30 versus 0%). In HKINs, p16 was more frequently positive than in LKINs (p=0.003; power = 49%) and SCCs (p=0.03; power = 53%). HKINs showed more frequent transepidermal p16 and p53 staining than LKIN lesions ($p < 0.001$;power $\geq$ 99%).

This study demonstrates that in KIN lesions and cutaneous SCCs, p16 expression is independent of p53 expression, and immune status, sun exposure, and histological diagnosis have no influence on this independency. Furthermore, HKIN lesions express significantly more p16 than LKINs and SCCs.

INTRODUCTION

Based on epidemiological, clinical, histopathological, and molecular studies, keratinocytic intraepidermal neoplasia (KIN) is considered an early step in squamous cell carcinoma (SCC) development [38]. Ultraviolet (UV) radiation is a prevailing factor implicated in the etiology of KIN and SCCs as evidenced by the high frequency of UV-related mutations in both the p53 and p16 tumor suppressor genes [110,129,187,188].

$p16^{INK4A}$ (p16) is encoded by the INK4a/CDKN2A gene located on chromosome 9p21[163]. P16 is a cyclin-dependent kinase inhibitor that specifically blocks the activity of cyclin-dependent kinases (CDKs) CDK4 and CDK6. By binding to CDK4, $p16^{INK4A}$ can inhibit the phosphorylation of pRB with subsequent inhibition of E2F release and arrest of the cell cycle in G1 phase and suppression of cell proliferation. Functional or structural loss of p16, could therefore lead to cell cycle propagation of potential genetically damaged cells and subsequent risk of tumor development. Links between the p16 and p53 pathways are present and mediated by $p14^{ARF}$, which is formed by an alternative reading frame in the *INK4a* gene, which is shared by p16 and p14 [42,163]. Therefore, loss of the *INK4a* gene disrupts two cell control pathways, one through $p16^{INK4A}$ /CDK4/6/pRB and the other through $p14^{ARF}$ /MDM2/p53.

P53 mutations are the most frequent genetic alteration in SCC of the skin with reported incidence of 63% in sporadic tumors and 48% in skin carcinomas of RTRs [129]. The majority of mutations bear an UV-signature.

UV-induced mutations of p16 have been reported in sporadic SCCs (≤24%)[110,188] and SCCs of xeroderma pigmentosum patients (33%). In the latter, statistically significant associations were found between the frequency of mutations in p53 and p16 and between the frequency of mutations in p53 and in p14 [187]. These positive associations were not found for the sporadic skin carcinomas [188]. Furthermore, experiments in cultured human keratinocytes have demonstrated different induction of both p16 and p53 by UVB radiation [37]. All these data suggest that the p53 and p16 proteins are part of parallel pathways controlling cell cycle and response to UV DNA damage in keratinocytes.

Immunocompromised patients, such as renal transplant recipients, have a markedly increased risk of developing KINs and SCCs of the skin, especially on sun exposed parts of the body [11,63]. In these patients, besides UV, immunosuppressive treatment and human papilloma virus (HPV) also are considered risk factors for the enhanced cutaneous carcinogenesis [43,63,212]. The multiplicity of skin cancers in these patients and these cancers' more aggressive behavior, with metastases in a considerable number of patients, suggest that the SCCs and their precursors in RTRs differ from those in immunocompetent individuals (ICIs) with regard to their clinicopathological behavior, which might be reflected in different expression of cell-cycle-associated proteins.

The aims of the present clinicopathological study were: (1) to assess a possible relation between two risk factors for cutaneous carcinogenesis (sun exposure and immune status) and the expression of p16 and p53 in patients with KIN and SCCs, (2) to assess whether the expression of p16 and p53 are statistically independent in SCCs and their precursors, and 3) to assess differences in p16 and p53 expression between KINs and SCCs.

MATERIALS AND METHODS

Patients and histopathology

For this retrospective study we retrieved formalin-fixed and paraffin-embedded skin excisions of 51 KIN lesions and 35 SCCs from 44 RTRs and 42 normal ICIs out of our archival material at the Department of Pathology, University Medical Center Nijmegen St.Radboud, Nijmegen, the Netherlands.

Histology of all lesions was reviewed by the same dermatopathologist. A KIN classification was applied to all dysplastic skin lesions according to the criteria for KIN grading proposed by Cockerell in 2000 [38]. In this grading system KIN I is histologically characterized by focal atypia of basal keratinocytes of the *lower one third* of the epidermis; KIN II lesions demonstrate (focal) atypia of keratinocytes in the *lower two thirds* of the epidermis (often with hyperkeratosis and sparing or some involvement of acrotrichia and acrosyringia and/or presence of acanthosis and/or basal budding). KIN III lesions are characterized by a diffuse atypical keratinocytic proliferation involving the *full thickness* of the epidermis. We considered KIN I and II to be low-grade KIN (LKIN), corresponding to actinic keratoses (AKs). KIN III was considered to be high-grade intraepidermal neoplasia (HKIN), corresponding to Bowen's disease or squamous cell carcinoma *in situ* [68]. This separation was made because clinical behavior and, subsequently, treatment of AKs is generally different from Bowen's disease.

SCCs were classified according to their most poorly differentiated region and a subclassification in three categories of *well, moderately* and *poorly* differentiated was used [131].

Immunohistochemistry

Immunohistochemical analysis was performed on all lesions using a standard avidin-biotin-peroxidase complex system with diaminobenzidine as the chromogen. In brief, 4-μm-thick paraffin sections were deparaffinized, hydrated, and washed in phosphate buffered saline.

The antibodies used, pretreatment and dilutions are listed in Table 1. For p16 immunostaining we used the $p16^{INK4A}$ Ab-4, clone 16PO4 or JC2 (Neomarkers, Fremont, CA). With this antibody we have experience in (pre)malignant lesions of the uterine cervix with comparable results, as reported in literature [103,167]. Furthermore, this is one of the few p16 antibodies with a known epitope (aa1-32 of p16), whereas the epitope has not yet been determined for most other p16 antibodies.

After incubation with primary antibodies, sections were incubated for 30 minutes with biotinylated horse anti-mouse (1:200, Vector Laboratories, Burlingame, CA), followed by 45 minutes' incubation with avidin-biotin complex (1:50, Vector laboratories).

Sections were counterstained with Mayer's hematoxylin.

Table 1. Antibodies with used dilution, pretreatment, incubation time, and temperature.

Antibody	Antigen	Source	Dilution	Pretreatment	Temperature
DO7	p53	Neomarkers, Fremont CA	1:400	microwave	4^0C overnight
$P16^{INK4}$ Ab-4 clone 16PO4 or JC2	aa 1-32 of $p16^{INK4a}$	Neomarkers, Fremont CA	1:100	microwave	4^0C overnight

Quantification of immunohistochemical results

Immunoreactivity was scored semiquantitatively: 0 (negative), 1+ (≤10% of lesional cells positive), 2+ (10-50% of lesional cells positive), or 3+ (> 50% lesional cells positive).
In addition, in positive-staining KIN lesions, localization of p16 and p53 immunoreactivity in the epidermal cell layers was assessed: 0 = only basal layer positivity, 1 = positivity confined to basal one third of epidermis, 2 = positivity confined to basal two thirds of epidermis, or 3 = transepidermal positive staining.
Scoring was performed without knowledge of patient history.

Statistics

The association between the presence of p16- and p53-expression was assessed with 2 x 2 contingency tables. The presence of p16 and p53 expression is statistical independent if the conditional odds ratios between p16 and p53 expression do not differ significantly from 1. On the basis of the two presently studied risk factors (sun exposure and immune status), four risk groups were discerned, and on the basis of the histological diagnosis, patients were divided in three groups (LKIN, HKIN and SCC). It may be possible that the association between the presence of p16 and p53 expression depends on the risk factors and/or the severity of the skin lesion. Therefore, analyses of stratified 2 x 2 tables were performed. The associations between the presence of p16 and p53 expression for the separate strata were tested with the Fisher's exact test. The Breslow-Day statistic was used to test whether the associations, defined in terms of conditional odds ratios, are equal for the different strata (testing the homogeneity of odds ratios). The Mantel-Haenszel estimator was used as a common odds ratio for the several strata. This estimator was used to test whether the estimated common odds ratio differed significantly from 1. If the Breslow-Day test for homogeneity of odds ratios and Mantel-Haenszel test for conditional independence resulted in P values > 0.05, we concluded that the presence of p16-expression is statistically independent on the expression of p53 and that this independency is not influenced by sun-exposure and immune status or histological diagnosis.
The differences between LKIN, HKIN, and SCC with regard to the extent of p53 and p16 expression in relation to the risk factors were analyzed by means of I X J two-way contingency tables. The same analyses were also used to assess the differences between well, moderately and poorly differentiated SCCs with regard to extent of p53 and p16 expression in relation to the two risk factors. In these analysis significance was set at $P \leq 0.05$.
The SPSS exact tests, available in SPSS 10.0 for Windows, were used instead of the large-sample approximations to obtain the exact P values. Detailed information concerning the aforementioned statistical procedures is given in the literature[2].
Because of relatively small patient numbers, power analysis was performed using Power and Precision 2 (http://www.Power-Analysis.com). Fisher's exact method or the Casagrande and Pike method (approximation of Fisher's exact test) was used to compute power for the Fisher's exact tests performed.

RESULTS

Patients

Table 2 summarizes the data from all analyzed lesions.
Lesions from the head and neck region, hands, and forearms were considered as sun-exposed: 65 of the examined 86 lesions came from sun-exposed sites (75%), 18 cases (21%), from nonexposed sites; and in 3 cases, the localization was unknown (4%).

Table 2. Summary of cases.

All cases n=86	M: F	Age (mean±SD)	Lesion type and number
RTRs n=44	24:20	55.3±9.3	LKIN n=10 HKIN n=14 SCC n=20
ICIs n=42	21:21	72.5±11.4	LKIN n=13 HKIN n=14 SCC n=15

Dependency of p16 expression on p53 expression in relation to the four combinations of the two studied risk factors (sun exposure and immune status)

The frequencies of cases with regard to the expression of p16 and p53 in the 4 different risk categories are given in Table 3 for 83/86 lesions (of 3 lesions, localization and therefore sun exposure were unknown). The Fisher's exact test disclosed that in each of the four different risk groups the expression of p16 and p53 is statistically independent. The Mantel-Haenszel test disclosed that the odds ratios in the four risk groups did not significantly differ from 1 (P = 0.82), and the Breslow-Day test disclosed that the four odd ratios for these risk groups did not differ significantly (P = 0.32).
Therefore, the expression of p16 and p53 are conditionally independent and homogeneous for the four risk groups examined in this study.
In RTRs, 10/44 (23%) cases were p53-/p16+, compared to 5/42 (12%) cases in ICIs and these differences were also not statistically different (P = 0.26; power = 53%).

Table 3. The four expression patterns for p16 and p53 in relation to the 4 combinations of the 2 studied risk factors, transplantation status and sun exposure, in 83 of the 86 patients (in 3, specimen localization of the lesion was unknown).

Risk factors	P16-/p53- No. (percentage)	P16-/p53+ No. (percentage)	P16+/p53- No. (percentage)	P16+/p53+ No. (percentage)	Fisher test two-tailed p-value	Power (Casagrande and Pike)
Sun-/RTR- (N=9)	1 (11%)	2 (22%)	0	6 (67%)	0.3	20%
Sun-/RTR+ (N=9)	0	1 (11%)	2 (22%)	6 (67%)	>0.9	48%
Sun+/RTR- (N=32)	1 (3%)	9 (28%)	5 (16%)	17 (53%)	0.6	61%
Sun+/RTR+ (N=33)	2 (6%)	7 (21%)	7 (21%)	17 (52%)	>0.9	72%
TOTAL (N=83)	N=4 (5%)	N=19 (23%)	N=14(17%)	N=46 (55%)	0.8	96%

Dependency of p16-expression on p53-expression in relation to histological diagnosis (of LKIN, HKIN, and SCC)

The frequencies of the 4 different expression patterns of p16 and p53 for patients with KIN and SSC are given in Table 4. This table discloses that absence of both p16 and p53 was only present in 4/86 (5%) specimen, whereas combined expression of p53 and p16 was most prevalent in 48/86 (56%) cases.

Table 4. The expression patterns for p16 and p53 in relation to lesion type for the whole group of 86 patients.

Lesion type	P16-/p53- No. (percentage)	P16-/p53+ No. (percentage)	P16+/p53- No. (percentage)	P16+/p53+ No. (percentage)	Fisher test two-tailed p-value	Power (Casagrande and Pike)
LKIN (N=23)	2 (9%)	8 (35%)	5 (22%)	8 (35%)	0.4	20%
HKIN (N=28)	0	2 (7%)	6 (21%)	20 (71%)	>0.9	68%
SCC (N=35)	2 (6%)	9 (26%)	4 (11%)	20 (57%)	>0.9	76%
TOTAL (N=86)	N=4 (5%)	N=19 (22%)	N=15 (17%)	N=48 (56%)	0.8	98%

The Fisher's exact test disclosed that in LKIN, HKIN and SCC the expression of p16 and p53 are statistically independent.

The Fisher's exact test disclosed that in low-grade KIN, high-grade KIN, and SCC, the expressions of p16 and p53 are statistically independent. The Mantel-Haenszel test disclosed that the odds ratios in these three diagnostic groups did not significantly differ from 1 (P = 0.6), and the Breslow-Day test disclosed that the three odd ratios for LKIN, HKIN, and SCC did not differ significantly (P = 0.62). Therefore, the expression of p53 and p16 are conditionally independent and homogeneous for LKIN, HKIN, and SCCs (also see Fig.1 for illustration of immunohistochemical staining combinations for p16 and p53).

The extensiveness of the p16 expression in KINs and SCCs

P16 staining was cytoplasmic, or was both cytoplasmic and nuclear. We considered both strong cytoplasmic staining as well as nuclear staining to be positive reaction, in analogue to previous studies on the uterine cervix [99,103,167]. Normal epidermis showed only positive staining in melanocytes, with absent staining in keratinocytes (Fig.2A).

In total, 63/86 (73%) lesions were p16 positive, and 23/86, negative (27%; Table 5). In our series, none of the lesions showed exclusive nuclear staining. The combined nuclear and cytoplasmic p16 staining was most prevalent and was present in 54 cases (63%). Only nine cases (10%) showed exclusively cytoplasmic staining (three LKIN lesions and six SCCs).

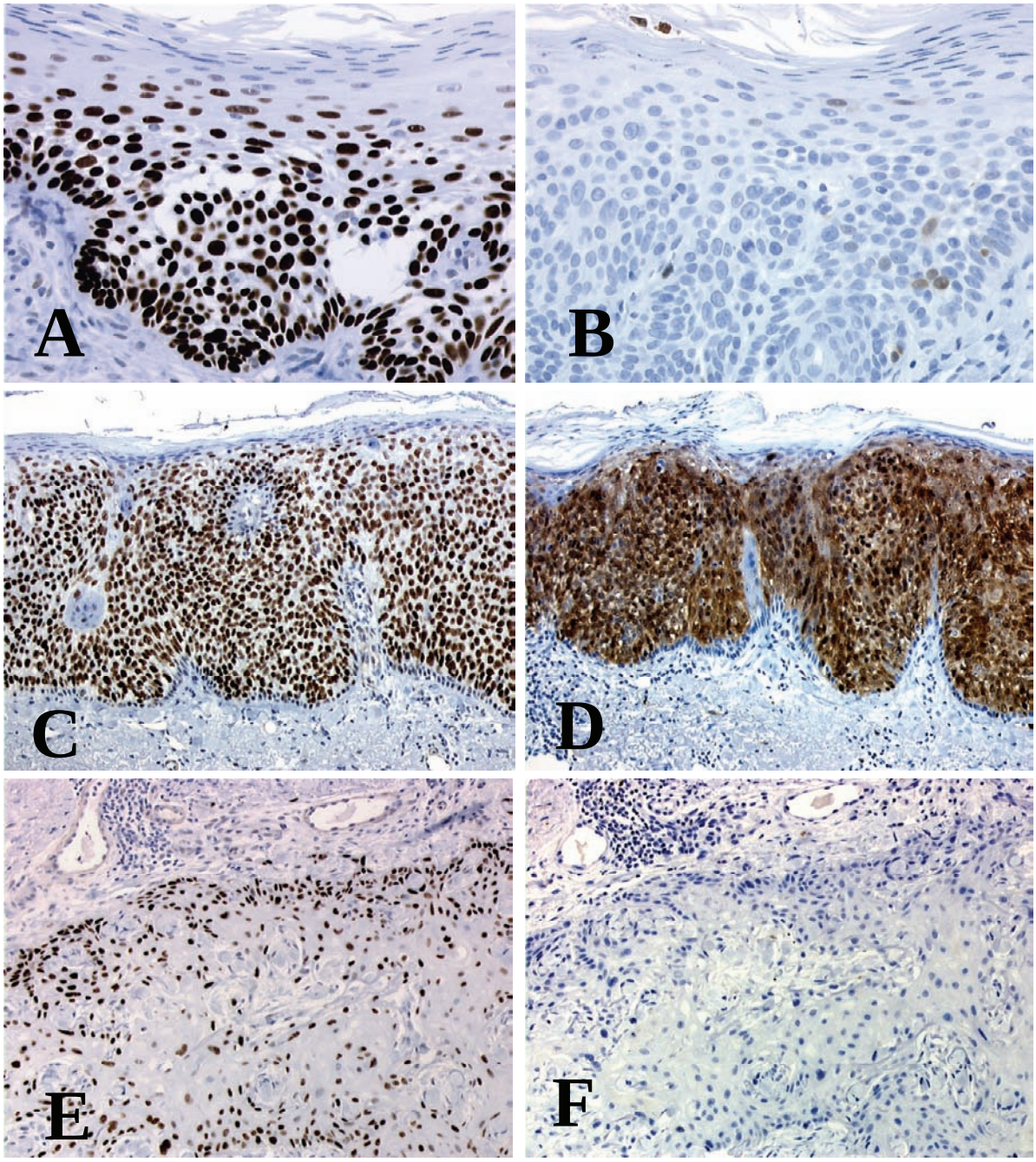

FIGURE 1
A-B: positive p53 (**A**) with absent $p16^{INK4A}$ (**B**) expression in KIN II of an immunocompetent control patient. P53 staining is restricted to the dysplastic basal two thirds of the epidermis.
C-D: strong transepidermal expression of p53 (**C**, nuclear staining) and $p16^{INK4A}$ (**D**, nuclear and cytoplasmic) in a KIN 3 lesion (high grade KIN) of an immunocompetent patient.
E- F: positive nuclear p53 (**E**) and absent p16 (**F)** staining in a squamous cell carcinoma of an immunocompetent individual.

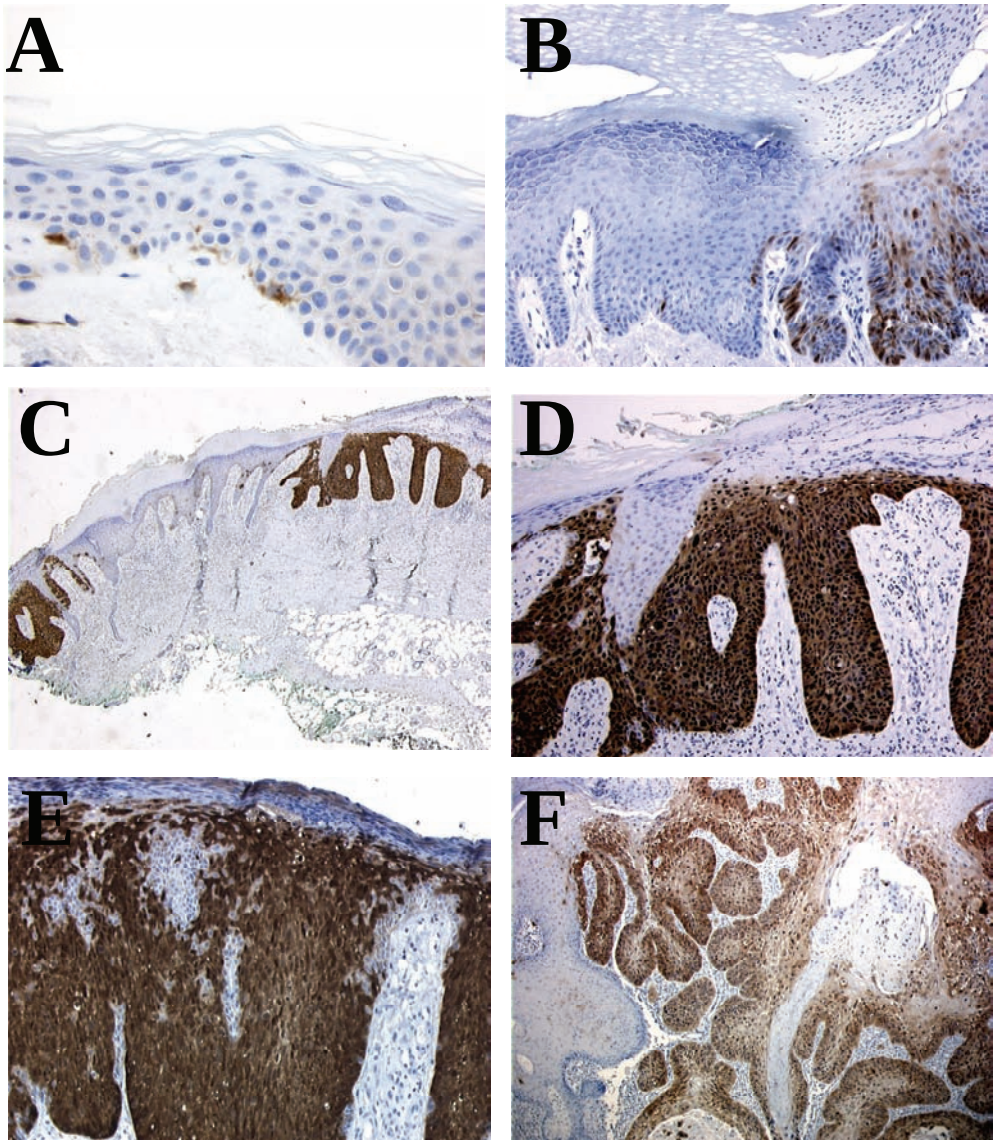

FIGURE 2
P16 expression patterns in normal skin (**A**) with only dendritic melanocytes staining positive; in LKIN of renal transplant recipient (RTR) showing more focal p16 staining in basal two thirds of the epidermis (**B**); and in HKIN of RTR with diffuse and strong, nuclear and cytoplasmic, transepidermal p16 staining, with sharp demarcation from normal skin (**C-E**), and in a SCC of RTR (**F**).

Table 5. P16 expression in LKIN, HKIN, and SCCs in the patient group as a whole.

	LKIN N=23	HKIN N=28	SCC N=35	Total N=86
P16 negative	10 (43%)	2 (7%)	11 (31%)	23 (27%)
P16 cytopl positive	3 (13%)	0	6 (17%)	9 (10%)
P16 combined nucl. & cytopl positive	10 (43%)	26 (93%)	18 (51%)	54 (63%)
Basal 1/3	5	0		
Basal 2/3	5	0		
transepidermal	3	26		

In KIN lesions further specified by location. As for p53 (Table 6), strongly significant more frequent transepidermal staining is present in HKIN compared to LKIN ($P < 0.001$).

Figure 3, A-B, illustrates the percentage of p16-positive-stained lesional cells in LKIN, HKIN, and SCCs for RTRs and ICIs, respectively. Statistical analysis disclosed no significant differences between ICIs and RTRs concerning the frequencies of the four p16 staining

categories for LKIN ($P = 0.22$; power = 68%), HKIN ($P = 0.46$; power > 66%), and SCC ($P = 0.44$; power = 74%).
In fig.3E the extensiveness of p16 expression in well, moderately and poorly differentiated SCC, pooled over ICIs and RTRs, are given. The differences between the well, moderately and poorly differentiated tumors were not significant (p=0.20).

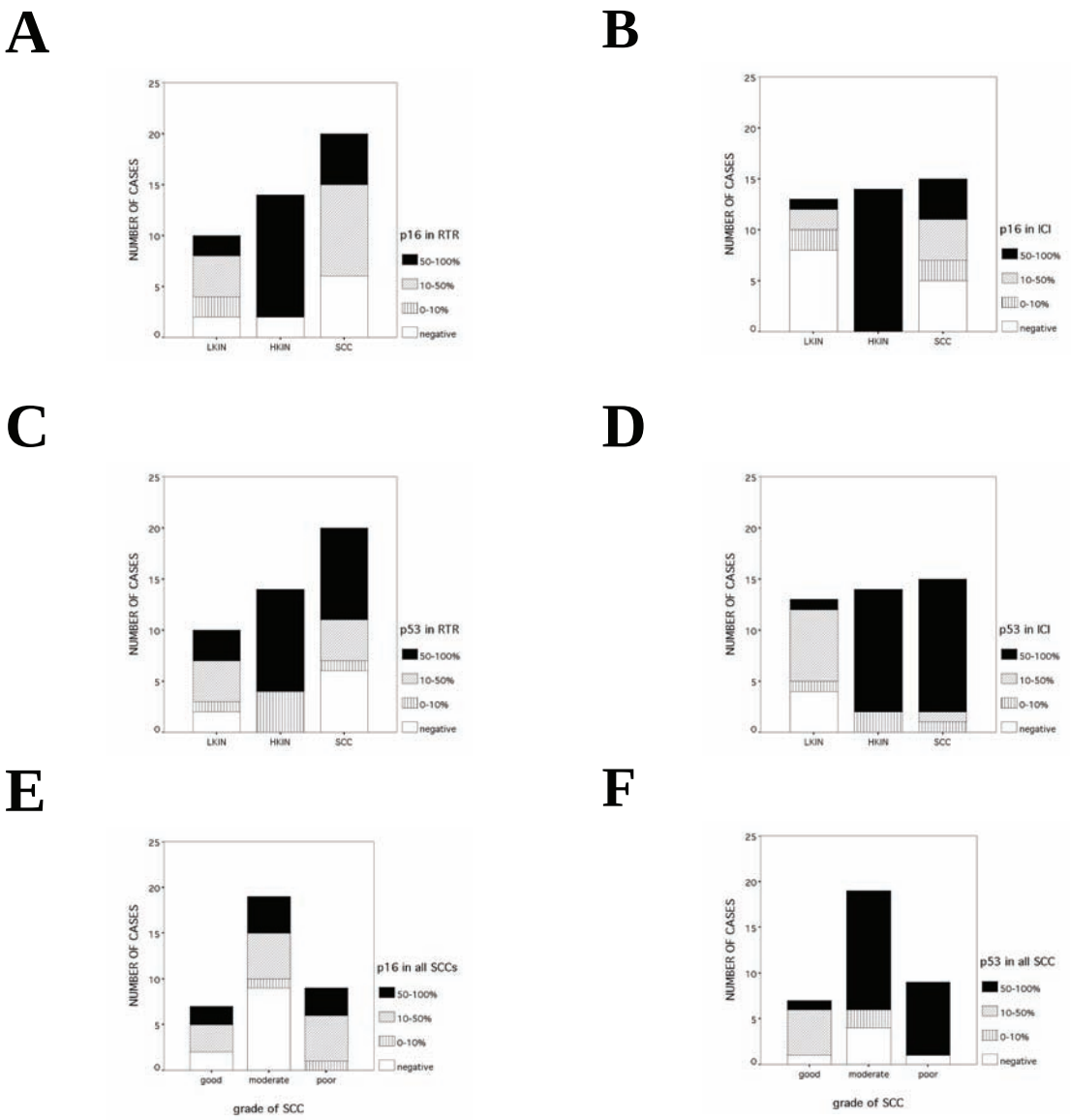

FIGURE 3
Percentages of p16 (**A, B**) and p53 (**C, D**) positive lesional cells in LKIN, HKIN, and SCCs for the RTRs and the ICIs, respectively.
In **E** and **F** the percentage of positive lesional cells for p16 and p53, respectively, are given in all combined SCCs divided in well, moderately and poorly differentiated tumors.

Table 5 shows for KIN lesions the epidermal localization of p16 staining and discloses that HKIN lesions were more frequently positive than LKIN lesions ($P = 0.003$; power = 49%) and SCCs ($P = 0.03$; power = 53%). Furthermore, strongly significant, more frequent transepidermal p16 staining was present in HKIN compared to LKIN ($P < 0.001$;power = 100%); epidermal localization of p16 staining proved strongly correlated with KIN grade ($r = 0.83$; $P < 0.001$; power = 100%).

In all p16 expressing HKIN lesions, a transepithelial expression was observed, whereas in only 3 of 13 LKIN lesions (23%) such an expression was found. The LKIN lesions with a transepithelial expression were morphologically classified as moderate dysplasia/KIN II. In HKIN lesions, p16 staining was continuously and diffuse present throughout all epidermal layers, with sharp demarcation between the normal and dysplastic epidermis (fig.2, C-E).

The extensiveness of the p53-expression in KINs and SCCs

P53 staining was nuclear. Normal epidermis showed only slight nuclear staining in a few scattered basal keratinocytes.

In total, 74/86 lesions (86%) were p53 positive and 12/86 (14%) were p53 negative (Table 6). Percentages of p53-positive lesional cells in LKIN, HKIN, and SCCs for the RTRs and the ICIs, respectively, are illustrated in Figure 3, C-D. For LKIN and HKIN no significant differences were found between ICIs and RTRs with regard to the frequencies of the four p53-immunostaining categories ($P \geq 0.65$; power = 72% and 61% respectively). Only for SCCs was a significant difference in the prevalence of p53 positivity present between RTRs and ICIs ($P = 0.02$; power = 47%), due to a significantly higher frequency of SCC without p53 expression in RTR (6/20=30%) than in ICI (0/15=0%). The extensiveness of p53 expression in SCC was highly associated with grade of the SCC. The percentage of SCCs with > 50% positive lesional cells increased with decrease in differentiation grade (Fig. 3F). In well, moderately and poorly differentiated SCCs respectively 14%, 68%, and 89% of the tumors showed >50% positive tumor cells. The prevalence of well, moderately and poorly differentiated SCCs in ICIs and RTRs was not significantly different ($P = 0.26$; power =66%). Therefore, the above-described significantly higher frequency of p53-negative SCCs in RTRs is not attributable to different prevalence of tumor grades between both groups.

As for p16, in KIN lesions, strongly significant, more frequent transepidermal p53 staining was present in HKIN lesions compared to LKIN lesions ($P < 0.001$: Table 6; power = 99%). Transepithelial staining was observed in 22/28 (79%) of p53-positive HKIN, and in only 1/17 (6%) of p53-positive LKIN lesions. Absence of p53 staining was exclusively found in LKIN, and p53 expression was positively correlated with KIN grade ($r = 0.42$; $P < 0.001$; power = 43%).

Table 6. P53 expression in LKIN, HKIN, and SCCs in the patient group as a whole, specified by location in KIN lesions.

	LKIN N=23	HKIN N=28	SCC N=35	Total N=86
P53 negative	6 (26%)	0 (0%)	6 (17%)	12 (14%)
P53 positive	17 (74%)	28 (100%)	29 (83%)	74 (86%)
Basal layer	1	6		
Basal 1/3	8	0		
Basal 2/3	7	0		
Transepidermal	1	22		

Strongly significant more frequent transepidermal staining is present in HKIN compared to LKIN ($P < 0.001$).

DISCUSSION

The present study is the first regarding immunohistochemical expression patterns of p16 in KIN and SCCs of RTRs. RTRs develop multiple HPV-induced warts, AKs and non melanoma skin cancers, especially on sun-exposed areas, which are directly related to the extent and duration of immunosuppression [16,26,82,86,94,210]. In these immunosuppressed patients, it is thought that DNA repair and apoptotic mechanisms are abrogated by UV and HPVs and, along with the altered immune surveillance of these patients, might allow progression of benign HPV epidermal lesions to malignancy.

In this study we show that both p16 as well as p53 are frequently overexpressed in KIN and SCCs of both RTRs and ICIs and that the expression of p16 is independent of p53 expression. This independency on the protein level parallels previous molecular findings in sporadic SCCs, in which p16 and p53 mutations proved to be independent events [188]. Recently, it was also demonstrated that the p16 and the p53 protein were differently modulated by UV depending on the dose and regimen [37]. These latter *in vitro* results strongly infer that both proteins are part of parallel pathways controlling response of keratinocytes to UV DNA damage. In our clinicopathological study, we found that the expression of p16 and p53 in skin lesions for sun exposed and non-sun exposed areas of the body did not differ, suggesting that the p16-p53 parallelism is not influenced by UV radiation.

In addition, we found that p16 and p53 expression were conditional independent of RTR status. We found that combined p16-p53 expression was most prevalent (46/86 lesions, 56%). Together, these observations suggest that both p16 and p53 are strongly involved in epidermal carcinogenesis but that expression levels of both proteins are independent of etiologic factors (sun exposure and immune status). Therefore, RTRs seem to use comparable pathways in skin cancer development as ICIs despite differences in immune status, but their susceptibility and rate to develop SCCs might well be determined by the deficient immune system.

Immune status was associated with p53 expression in SCCs ($P = 0.02$) because of a higher prevalence of p53-negative cases in RTRs compared to ICIs (30% *versus* 0%). Therefore, p53 seems differently involved in late stages of epidermal neoplasia in transplant recipients. Although in the present study, we did not perform PCR analysis for HPV status, one could speculate that the higher incidence of p53 negative tumors in RTRs suggests involvement of (oncogenic) HPV in the RTR group.

In cervical lesions, p53 is inactivated due to the transforming activities of the E6 viral oncoprotein of the mucosal high-risk HPV types, for instance HPV16 [99,192]. Overexpression of p16 in the (pre)malignant lesions of the uterine cervix is assumed to be the consequence of the inactivation of pRb by E7 of high-risk HPV types [Sano, 1998 #448}. So far, the knowledge on transforming properties of E6 and E7 of cutaneous HPV types is limited. In contrast to cervical cancer, in which high-risk HPV DNA becomes integrated in the host genome, in NMSCs containing presumed oncogenic HPV types, HPV integration is rare, and it was shown that E6 oncoprotein of, for instance, the cutaneous epidermodysplasia verruciformis-associated HPV38 could not degrade p53 protein [34,121]. Surprisingly, HPV 38 E6/E7-positive cells also did not express p16, in contrast to HPV 16 E6/E7-positive cells, whereas both HPV 38 and HPV 16 E7 had similar biological properties and both were able to inactivate pRb[34]. We were unable to demonstrate statistical linkage of the combined staining pattern with p53 negativity and p16 positivity (indicative of oncogenic HPV involvement) to the RTR population ($P = 0.26$). Therefore, based on the immunoexpression profiles for p53 and p16 presently found, and because of the incomplete knowledge of the interactions of cutaneous E6 and E7 proteins with p53 and p16, the role of HPV in cutaneous carcinogenesis in RTRs remains speculative.

So far only a few studies have evaluated p16 immunoexpression in cutaneous SCCs and precursors. Hodges and Smoller [87] described p16 positivity in all 10 AKs, SCC *in situ* lesions, and SCCs studied, with the use of a different antibody (G175-405, Pharmingen, San Diego, CA) but a comparable scoring system, although cellular localization (either cytoplasmic and/or nuclear staining) was not further specified. Transepidermal staining was only present in SCC *in situ*, comparable to our results in HKINs. In contrast, we found statistically significant more p16 positivity in HKIN lesions (93%) than in LKIN and SCCs (57% and 69% respectively, $P = 0.003$ and $P = 0.03$). Mortier *et al.* [140] found p16 expression in 66% of AKs and only 10% of SCCs, without specification of the used scoring method. In contrast to our data and those of Hodges and Smoller, those investigators also describe p16 expression in normal skin with the use of their antibody (G175-405, Pharmingen).

Combined mutation analysis with immunohistochemistry for p16 so far was only performed by Chang *et al.* [35] , albeit on a very small number of SCCs (6 cases): two of six SCCs showed positive nuclear staining for p16 (anti-p16 antibody, C-20; Santa Cruz Biotechnology), and four of six SCCs were negative. In all cases, wild type p16 was present and no mutations could be demonstrated, implicating that immunohistochemical findings cannot directly be correlated to underlying molecular alterations. These conflicting results with respect to p16 staining in SCCs of skin and precursors seem related to usage of different p16 antibodies, often-small patient numbers, and different immunohistochemical scoring methods. The question arises as to what these different p16 antibodies actually recognize.

In imitation of immunohistochemical studies on p16 expression in (pre)malignant lesions of the uterine cervix, we considered both nuclear and strong cytoplasmic p16 staining as a positive reaction [99,103,167]. Yet we have to admit that up to now, the significance of cytoplasmic p16 staining is still unclear, and conflicting data have been presented with respect to the specificity of cytoplasmic p16 staining in adenocarcinomas [71,178]. In our series, none of the lesions showed exclusive nuclear staining: the combined nuclear and cytoplasmic p16 staining was most prevalent: 54 cases (63%) and only 9 cases (10%) showed exclusively cytoplasmic staining (3 LKIN lesions and 6 SCCs). When considering only the cases with combined staining to be p16 positive, statistical analysis disclosed no effects on the presented conclusions (data not shown).

In conclusion, according to our data, squamous cell carcinogenesis in RTRs seems to occur along comparable pathways as in ICIs, with independent parallel involvement of both the p53 and the p16 pathway. The present study demonstrates that p53 and p16 immunoexpression are conditionally independent of the two studied risk factors, transplantation status and sun exposure. The expression of p16 proved conditionally independent of the p53 expression in KIN and SCCs, and immune status and sun-exposition had no influence on this conditional independence. Future studies combining immunohistochemistry and molecular data are needed to elucidate the exact role of p16 in epidermal carcinogenesis.

2.2

P14 Expression and HPV in keratinocytic intraepidermal neoplasia (KIN) and cutaneous squamous cell carcinoma

Abstract

The tumor suppressor p14 forms a connection between the two main pathways governing cell growth, namely the p14-MDM2-p53 and p16-CDK4/6-RB pathway. Previously, we found high-grade keratinocytic intraepidermal neoplasia (HKIN) lesions to frequently overexpress the tumor suppressor proteins p53 and p16. The expression of p14 in KIN lesions has not been studied yet. In skin tumors sun exposition, immune suppression and HPV are important etiological factors.

Based on findings in cervical carcinogenesis, one could hypothesize that p14 and p16 would be overexpressed in HPV associated cases and based on in vitro studies inverse relations between p53 and p14 expression could be expected.

The aims of this study were to assess 1) whether the expression of p14, p16 and p53 are statistically independent in KINs and cutaneous squamous cell carcinomas (CSCCs), and 2) to assess a relation with etiological factors for cutaneous carcinogenesis (sun exposure and immune status), and 3) to determine the presence of mucosal HPV types in KINS and CSCCs.

Immunostaining for p14, p16 and p53 was performed on paraffin embedded sections of 22 low grade KIN (LKIN) lesions, 49 HKINs, and 34 CSCCs from 52 RTRs and 53 ICIs. HPV detection was performed on consecutive sections using a short PCR fragment (SPF-LiPA) assay, allowing for the simultaneous detection, and typing of 25 mucosal HPV genotypes.

P14 was expressed in a total of 42/105 lesions (40%). In LKIN lesions 22% was p14 positive, in HKINs 57%, and in CSCCs 50% of cases were p14 positive (p=0.05). P14 expression proved independent of the expression of both p53 and p16, and immune status, sun-exposure and histological diagnosis had no influence on this independency.

Only 2/105 specimens, both HKIN lesions in ICIs, contained HPV X and none of the 25 known mucosal genotypes was detected. Due to this low number of HPV positive cases no correlations with tumor suppressor protein expression could be studied.

INTRODUCTION

Cell growth is controlled by two main pathways, one involving the retinoblastoma protein (pRb), regulating exit from the G1 phase of the cell cycle, and one involving the p53 protein that induces growth arrest or apoptosis in response to cellular stress or DNA damage.
pRb is inactivated by phosphorylation, and inactivation of RB releases the E2F1 transcription factor from the RB complex. This factor regulates the expression of many genes involved in entry and progression through the S phase of the cell cycle.
Connections between the p53 and pRb pathways are provided by MDM2, by p21 and an additional important junction is provided by the INK4a-ARF locus located on human chromosome 9p21. This locus, by alternative transcripts encodes for two proteins $p16^{INK4a}$/p16 (exons 1α, 2 and 3), and $p14^{ARF}$ /p14 (exons 1β and 2).
Overexpression of p16 inhibits entry and progression into the S phase of the cell cycle by inhibition of CDK4 and 6, thereby preventing phosphorylation of RB and release of E2F. Loss of p16 may allow excessive activity of CDKs, promoting RB phosphorylation and proliferation of tumor cells.
Unlike p16, $p14^{ARF}$ (p14) does not bind to CDKs. P14 interacts with MDM2 and sequesters MDM2 in the nucleolus and enables p53 stabilization. P53 has an important function in cell cycle arrest and apoptosis. Also E2F1-induced activation of p53 is mediated by p14, which is transcriptionally activated by E2F1 [54,174]. This responsiveness of p14 to E2F1 makes p14 an important nexus between the pRB and p53 pathways. The p14-E2F1 interplay enables the cell to sense oncogenic stimuli transduced through the RB-pathway, such as p16 inactivation. P14 is also induced by oncogenes such as myc and ras, and some viral oncogenes.
Furthermore, p53 negatively regulates p14 creating a negative feedback loop between p14 and p53 [193], which explains the reported inverse correlation between p14 and p53 expression in human tumor cell lines [193].
Therefore p14 plays a central role in cell cycle control, by integrating all kind of stimuli and by targeting at least two of the key pathways in control of cell cycle, namely the p14-MDM2-p53 pathway and the p16-CDK4/6-RB pathway.
Previously, we have studied the expression of p16 and p53 in (pre)malignant epidermal tumors of renal transplant recipients (RTRs) and immunocompetent individuals (ICIs) [18]. At present, there is only one study regarding p14 expression in skin tumors [32].
From studies on cervical (pre)cancer, it is known that the three-tumor suppressors p53, p16 and p14, are all influenced by the presence of HPV. In cervical carcinogenesis, the E6 and E7 oncoproteins of high-risk HPV attribute to tumor development by inactivating p53 and Rb, leading to overexpression of p16 and p14 [166]. In skin tumors of both RTRs and ICIs, the presence of HPV DNA is reported, including high-risk mucosal HPV types [89,164,173], although data on frequency and types found are conflicting. We speculated that if high-risk mucosal HPV types indeed play a role in cutaneous carcinogenesis, comparable effects of HPV on expression of the tumor suppressors p14, p16, and p53 could be expected in skin tumors.
Immunocompromised patients, such as renal transplant recipients, have a markedly increased risk to develop KINs and SCCs of the skin [11,63]. In these patients besides UV, also immunosuppressive treatment and Human Papilloma Virus (HPV), are considered risk factors for the enhanced cutaneous carcinogenesis [43,63,212]. The multiplicity of skin cancers in these patients and their more aggressive behavior with metastases in a considerable number of patients, suggest that the SCCs and their precursors in RTRs differ from those in ICIs with regard to their clinic pathological behavior which might be reflected in different expression of cell-cycle associated proteins.
The aims of the present study were : 1) to assess whether the expression of p14 /p53, and p14 /p16 are statistically independent in CSCCs and their precursors, and 2) to assess a possible

relation with risk factor for cutaneous carcinogenesis (sun exposure and immune status), and 3) to determine the presence of mucosal HPV types in order to clarify the role of (high risk) mucosal HPV types in skin carcinogenesis.

MATERIALS AND METHODS

Patients and histopathology

For this retrospective study we retrieved 105 formalin fixed and paraffin embedded keratinocytic intraepidermal neoplasia (KIN) lesions and SCCs from immunocompetent individuals (ICIs) and renal transplant recipients (RTRs) out of our archival material at the Department of Pathology, Radboud University Nijmegen Medical Center, the Netherlands.
Histology of all lesions was revised by the same dermatopathologist. All dysplastic skin lesions were classified according to the proposed criteria for KIN grading by Cockerell in 2000 [38]. In this grading system KIN I is histologically characterized by focal atypia of basal keratinocytes of the *lower one third* of the epidermis; KIN II lesions demonstrate (focal) atypia of keratinocytes in the *lower two thirds* of the epidermis (often with hyperkeratosis and sparing or some involvement of acrotrichia and acrosyringia and/or presence of acanthosis and/or basal budding). KIN III lesions are characterized by a diffuse atypical keratinocytic proliferation involving the *full thickness* of the epidermis. We considered KIN I and II as low grade KIN (LKIN), corresponding to actinic keratoses (AKs). KIN III was considered as high-grade intraepidermal neoplasia (HKIN), corresponding to Bowen disease or squamous cell carcinoma in situ. This distinction was made since clinical behavior and subsequently treatment of actinic keratoses is generally different from Bowen's disease.
SCCs were classified according to their most poorly differentiated region and a sub classification in 3 categories of "well", "moderately" and "poorly" differentiated was used [131].

Immunohistochemistry

For p14, p16 and p53 immunohistochemistry, 4μm thick paraffin sections were deparaffinized, hydrated and washed in buffered saline phosphate (PBS). Microwave pretreatment consisted of 3 min. cooking in citrate buffer (0.01M, pH6.0) at 850 Watt, and 10 min cooking at 180 Watt. After preincubation with 20% normal goat serum for 10 min, sections were incubated with primary antibodies for 60 min at room temperature.
For detection of p14, Ab-2, clone 14PO2 , lot no 850P203 (Neomarkers, Fremont, CA, USA; dilution 1:200) was used,; for p53, Ab-5 (DO-7), lot no 186p306 (Neomarkers, Fremont, CA, USA; dilution 1:200) was used and for p16, we employed $p16^{INK4A}$ Ab-4, clone 16PO4 or JC2 , lot no 887P301 (Neomarkers, Fremont, CA, USA; dilution 1:500).
After rinsing in PBS, 15 min post-antibody blocking (powervision plus) was performed followed by 30 min incubation with Poly-HRP-anti-mouse/rabbit/rat IgG (ready-to-use) (Kit Powervision and Poly-HRP-AntiMs/Rb/Rt IgG Biotin-free, one component ready-to-use, Immunologic, Duiven, the Netherlands, Code DPVO-999HRP, Lot.no. 30210-410). For development, diaminobenzidine (DAB) was used as the chromogen.
Sections were briefly counterstained with Mayer's haematoxylin.

Quantification of immunohistochemical results

P16 and p53 immunoreactivity was scored semi-quantitatively as previously described [18], and the same method was applied for scoring p14 immunoreactivity. In brief, immunoreactivity was scored semi-quantitatively: 0 (negative), 1+ (up to 10% of lesional cells positive), 2+ (10-50% positive lesional cells), or 3+ (> 50% positive lesional cells). In addition, in positive staining KIN lesions, localization of p16 and p53 immunoreactivity in the epidermal cell layers was assessed: 0= only basal layer positivity, 1=positivity confined to basal 1/3 of

epidermis, 2=positivity confined to basal 2/3 of epidermis or 3= transepidermal positive staining. Scoring was performed without knowledge of patient history.

HPV detection and genotyping by SPF_{10}-LiPA

A single 4-μm thick flanking tissue section obtained from the same tissue specimen as was used for the immunohistochemical staining, was put into a reaction tube and incubated overnight at 56˚C in 200 μl of 10 mM Tris-HCl with 1 mM EDTA, 0.2% Tween-20, and proteinase K (0.3mg/ml). Proteinase K was inactivated by 10 min incubation at 100˚C. The sample was centrifuged for 10 min at 11.000 rpm and 10 μl was directly used for PCR analysis. A water blank control was processed with each batch of ten samples. HPV detection was performed using a short PCR fragment (SPF_{10}-PCR) assay [104,134]. The SPF-PCR system amplifies a 65 bp fragment of the L1 open reading frame, allowing for detection of at least 43 HPV types. In case of positive PCR result, subsequent HPV genotyping was performed via a reverse hybridization line probe assay (LiPA), allowing for simultaneous typing of the following 25 genotypes of HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74. This combined SPF_{10}-LiPA system for detection and genotyping of HPV has previously been described in detail[134]. This HPV detection test is highly sensitive, specific, and reproducible and has been clinically validated [104,134,165]. All specimens were processed separately to avoid cross-contamination.

Statistics

In this study, the data were analyzed by non-parametric statistical procedures because the Lilliefors test for normality disclosed that the percentage of p16, p14 and p53 expression were not Gaussian distributed.

The association between the presence of p14- and p53-expression and between the presence of p14- and p16-expression was assessed with 2x2 contingency tables. The presence of p14 and p53 or p16 expression is statistical independent if the conditional odds ratios between p16- and p53-expression do not differ significantly from 1. On the basis of the two presently studied risk factors (sun exposure and immune status) four risk groups were discerned, and on the basis of the histological diagnosis patients were divided in three groups (LKIN, HKIN and SCC). It may be possible that the association between the presence of p16- and p53-expression depends on the risk factors and/or the severity of the skin lesion. Therefore, analyses of stratified 2x2 tables were performed. The associations between the presence of p16- and p53-expression for the separate strata were tested with the Fisher exact test. The Breslow-Day statistic was used to test whether the associations, defined in terms of conditional odds ratios, are equal for the different strata (testing the homogeneity of odds ratios). The Mantel-Haenszel estimator was used as a common odds ratio for the several strata. This estimator was used to test whether the estimated common odds ratio differed significantly from 1. If Breslow-Day test for homogeneity of odds ratios and Mantel-Haenszel test for conditional independence resulted in p-values greater than 0.05, we conclude that the presence of p14-expression is statistically independent on the expression of p53 or p16 and that this independency is not influenced by sun-exposure and immune status or histological diagnosis.

The difference between LKIN, HKIN, and SCC with regard to the extent of p14 expression was analyzed by means of *I X J* contingency tables. In these analysis significance was set at $p \leq 0.05$.

The SPSS exact tests, available in SPSS 10.0 for Windows, were used to obtain the exact p-values instead of the large-sample approximations for the p-values. Detailed information concerning the afore mentioned statistical procedures are given in the literature[2].

RESULTS

Patients

Table 1 summarizes the data from all analyzed lesions.
Lesions from the head and neck region, hands, and forearms were considered as sun-exposed sites: 75 of the 105 examined lesions came from sun-exposed sites (71%), 30 cases from nonexposed sites (29%).

Table 1. Data of all analyzed lesions.

	LKIN	HKIN	SCC	Total
ICI	6 (11%)	30 (58%)	16 (31%)	53
RTR	16 (30%)	19 (36%)	18 (34%)	52
Total	22 (21%)	49 (47%)	34 (32%)	105

LKIN= low-grade keratinocytic intraepidermal neoplasia, HKIN= high-grade intraepidermal neoplasia, ICI= immunocompetent individual, RTR= renal transplant recipient.

Dependency of p14 expression on p53 expression in relation to the four combinations of two studied risk factors (sun exposure and immune status)

The frequencies of cases with regard to the expression of p14 and p53 in the 4 different risk categories are given in ***Table 2***. The Fisher's exact test disclosed that in each of the four different groups the expression of p14 and p53 is statistically independent. The Mantel-Haenszel test disclosed that the odds ratios in the four risk groups did not significantly differ from 1 ($p=0.1$) and the Breslow-Day test disclosed that the four odds ratios for these risk groups did not differ significantly ($p=0.7$).
Therefore the expression of p14 and p53 are conditionally independent and homogeneous for the 4 risk groups examined in this study.
Single positivity (inverse relation between p14 and p53 expression) for one of the markers was present in 49/105 cases, while absence or presence of both markers was more prevalent and present in 56/105 cases. Most of the lesions showed a p14-/p53+ pattern (42/105 specimen, 40%). Analysis of the 2x2 tables for each of the 4 risk categories disclosed that expression of p14 and p53 are not dependent ($p>0.3$).

Table 2. The four expression patterns for p14 and p53 in relation to the 4 combinations of the 2 risk factors sun exposure and transplantation status, in all 105 lesions.

Risk factors	P14-/p53- Number (%)	P14-/p53+ Number (%)	P14+/p53- Number (%)	P14+/p53+ Number (%)	Fisher test two-tailed p- value
Sun-/ICI N=14	5 (36%)	6 (43%)	0	3 (21%)	0.3
Sun-/RTR N=16	4 (25%)	4 (25%)	2 (13%)	6 (37%)	0.6
Sun+/ICI N=38	4 (11%)	18 (47%)	1 (2%)	15 (39%)	0.4
Sun+/RTR N=37	8 (22%)	14 (38%)	4 (11%)	11 (30%)	0.7
Total N=105	21 20%	42 (40%)	7 (7%)	35 (33%)	

Dependency of p14 expression on p16 expression in relation to the four combinations of two studied risk factors (sun exposure and immune status)

The frequencies of cases with regard to the expression of p14 and p16 in the 4 different risk categories are given in ***Table 3***. As for p53, the Fisher's exact test disclosed that in each of the four different groups the expression of p14 and p16 is statistically independent. The Mantel-Haenszel test disclosed that the odds ratios in the four risk groups did not significantly differ from 1 (p=0.1) and the Breslow-Day test disclosed that the four odds ratios for these risk groups did not differ significantly (P=0.7).

Therefore also the expression of p14 and p16 are conditionally independent and homogeneous for the 4 risk groups examined in this study.

The combination of p14-/p16+ was most prevalent (41%), followed by double positivity for both markers. Analysis of the 2x2 tables for each of the 4 risk categories disclosed that expression of p14 and p16 are not dependent (p>0.5).

Table 3. The four expression patterns for p14 and p16 in relation to the 4 combinations of the 2 risk factors sun exposure and transplantation status, in all 105 lesions.

Risk factors	P14-/p16- Number (%)	P14-/p16+ Number (%)	P14+/p16- Number (%)	P14+/p16+ Number (%)	Fisher test two-tailed p-value
Sun-/ICI N=14	2 (14%)	9 (64%)	0	3 (22%)	>0.9
Sun-/RTR N=16	2 (12%)	6 (38%)	0	8 (50%)	0.5
Sun+/ICI N=38	8 (20%)	14 (37%)	4 (11%)	12 (32%)	0.5
Sun+/RTR N=37	8 (22%)	14 (38%)	3 (8%)	12 (32%)	0.5
Total N=105	20 (19%)	43 (41%)	7 (7%)	35 (33%)	

Dependency of p14 expression on p53 or p16 expression in relation to histological diagnosis of LKIN, HKIN and SCC

The frequencies of the 4 different expression patterns of p14 and p53 for patients with KIN and SCCs are given in ***Table 4***.

Table 4. The expression patterns for p14 and p53 in relation to the three diagnostic categories

Diagnosis	P14-/p53- Number (%)	P14-/p53+ Number (%)	P14+/p53- Number (%)	P14+/p53+ Number (%)	Fisher test two tailed p-value
LKIN n=22	7 (32%)	11 (50%)	1 (4%)	3 (14%)	>0.9
HKIN n=49	11 (22%)	17 (35%)	3 (6%)	18 (37%)	0.07
SCC n=34	3 (9%)	14 (41%)	3 (9%)	14 (41%)	>0.9
Total N=105	21 (20%)	42 (40%)	7 (7%)	35 (33%)	

The Fisher's exact test disclosed that in LKIN, HKIN and SCCs, the expressions of p14 and p53, are statistically independent.
The Mantel-Haenszel test disclosed that the odds ratios in these three diagnostic groups did not significantly differ from 1 (p=0.2), and the Breslow-Day test disclosed that the three odds ratios for LKIN, HKIN, and SCC did not differ significantly (p=0.5).
Therefore , the expression of p14 and p53 are conditionally independent and homogeneous for LKIN, HKIN, and SCC.

The frequencies of the 4 different expression patterns of p14 and p16 for patients with KIN and SCCs are given in ***Table 5***.

Table 5. The expression patterns for p14 and p16 in relation to the three diagnostic categories.

Diagnosis	P14-/p16- Number (%)	P14-/p16+ Number (%)	P14+/p16- Number (%)	P14+/p16+ Number (%)	Fisher test two tailed p-value
LKIN n=22	7 (32%)	11 (50%)	0	4 (18%)	0.3
HKIN n=49	7 (14%)	21 (43%)	0	21 (43%)	**0.02**
SCC n=34	6 (18%)	11 (32%)	7 (21%)	10 (29%)	>0.9
Total N=105	20 (19%)	43 (41%)	7 (7%)	35 (33%)	

The Fisher's exact test disclosed that in LKIN and SCCs, the expressions of p14 and p16, are statistically independent. In the HKIN group a weakly significant difference was present attributable to the strong correlation between p16 positivity and diagnosis of HKIN, see below.
The Mantel-Haenszel test disclosed that the odds ratios in these three diagnostic groups did not significantly differ from 1 (p=0.1), and the Breslow-Day test disclosed that the three odds ratios for LKIN, HKIN, and SCC only differed weakly significant (p=0.04), mainly attributable to HKIN lesions, as stated above.

The extensiveness of p14 expression in KINs and SCCs

Keratinocytes in normal skin were negative for p14. In all cases p14 staining was nuclear, often in a dotted ("nucleolar") pattern (***Figure 1***).

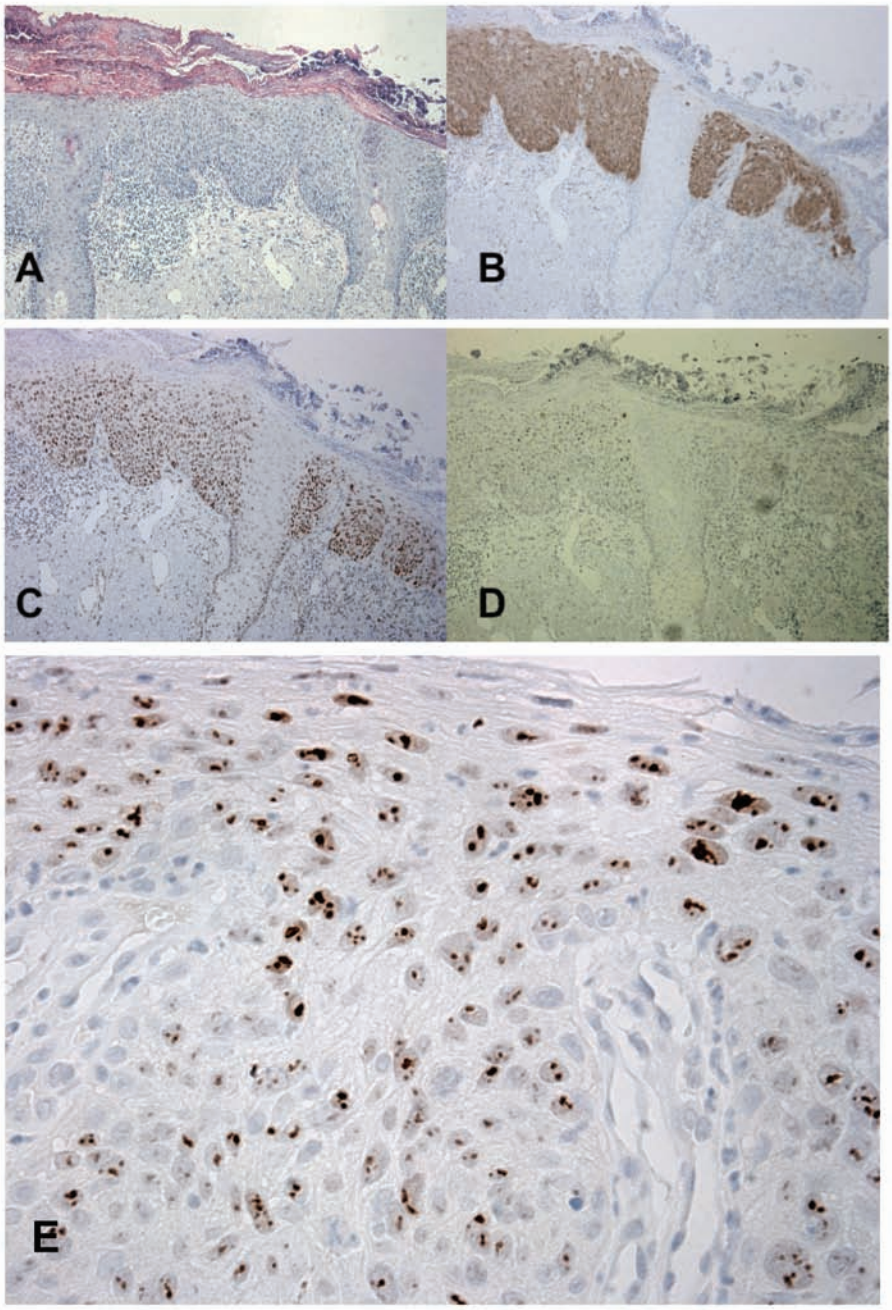

FIGURE 1.
A.HE of a HKIN lesion, which showed strong transepithelial staining with p16 (B), p53 (C), and p14 (D). As can be seen, staining for p14 is much more difficult to discern in tissue sections when compared to p16 and p53 using the same magnification. E. High magnification of the same case showing typical nucleolar staining for p14 (400x).

In total 42/105 (40%) cases were positive. In LKIN lesions 22% was p14 positive, in HKINs 57%, and in CSCCs 50% of cases were p14 positive. These differences in presence of p14 expression were weakly significant between the three diagnostic groups of LKIN, HKIN and SCC (***Table 6***).

Table 6. p14 expression in LKIN, HKIN, and SCC in the patient group as a whole

	LKIN	HKIN	SCC	total	p value (X sq)
P14+	4 (18%)	21 (43%)	17 (50%)	42 (40%)	0.05
P14-	18 (82%)	28 (57%)	17 (50%)	63 (60%)	
P16+	15 (19%)	42 (54%)	21 (27%)	78 (74%)	0.04
P16-	7 (26%)	7 (26%)	13 (48%)	27 (26%)	
P53+	14 (18%)	35 (46%)	28 (36%)	77 (73%)	0.3
P53-	8 (29%)	14 (50%)	6 (21%)	28 (27%)	

The percentage of positive lesional cells in LKIN, HKIN, and SCCs, for RTRs and ICIs respectively, are illustrated in ***Figure 2***. Statistical analysis disclosed no significant differences between ICIs and RTRs concerning the frequencies of the four-p14 staining categories for LKIN (p=0.2), HKIN (p=0.8), and SCCs (p=0.9).

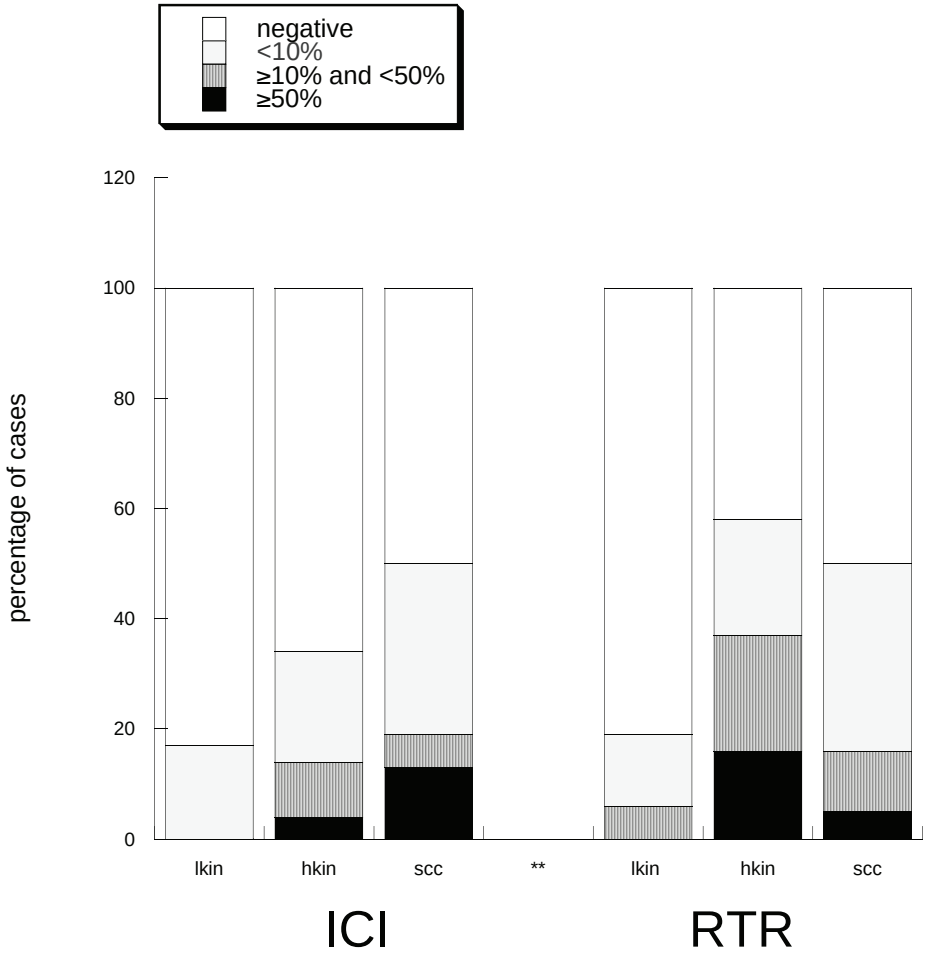

FIGURE 2
Percentage of cases in the group of ICIs and RTRs showing absent p14 staining, less than 10% positive cells, between 10-50% positive cells, and >50% p14 positive cells.

In general the number of p14 positive staining lesional in all three diagnostic categories was low.
Only 7/105 cases showed more than 50% p14-positive lesional cells; 4 of these cases were HKIN lesions, and 3 were SCCs.

The extensiveness of p53 expression and p16 expression in KINs and SCCs

We will only briefly report on findings with respect to p16 and p53 since results are comparable to previously described findings[18].

Normal skin showed only sporadic p53 staining in a few basal and suprabasal keratinocytes, and only p16 positivity in dendritic melanocytes.

In total 78/105 (74%) cases were p16 positive, in 76 cases both cytoplasmic and nuclear staining was present; in 2 cases only cytoplasmic staining was seen. There were a significantly higher number of p16 positive cases in the HKIN group.

P53 positivity was always nuclear and present in 77/105 (73%) lesions. There were no significant differences in number of p53 positive specimen between the three diagnostic groups.

Statistical analysis disclosed no significant differences between ICIs and RTRs concerning the frequencies of the four-p16 staining categories for LKIN ($p=0.2$), HKIN ($p=0.6$), and SCCs ($p=0.7$).

For p53, only for SCCs there was a weak significant difference in the prevalence of p53 positivity between RTRs and ICIs ($p=0.05$), due to higher frequency of p53 negative SCCs in RTRs, 33% (6/18 cases), than in ICIs (0/16 cases, 0%). KIN grade correlated strongly with localization of p53 and p16 staining in the epidermal layers ($r>0.7$, $p<0.0001$).

HPV detection and typing

Only 2/108 (1.9%) lesions contained HPV X DNA in SPF_{10}-LiPA. Neither one of them contained one of the 25 known mucosal genotypes. Both cases showed strong p16 staining (>95%), absent p14, and one case had low (15%) and the other case absent p53 staining. Due to the low number of specimen containing HPV DNA, no correlations between tumor suppressor expression and HPV were calculated. Both HPV containing specimen were HKIN lesions in ICIs.

DISCUSSION

The tumor suppressor p14 is thought to play a key role in cell cycle control, since p14 connects the two main pathways governing cell growth, namely the p14-MDM2-p53 and p16-CDK4/6-RB pathway. Both p14 and p16 both are products of one locus that has an unusual capacity to encode two structurally distinct proteins, the INK4a-ARF locus, and both have the potential to act as tumor suppressor and can be activated by oncogenes.

Studies on cell lines transfected with a vector encoding the HPV E6 oncoprotein have reported inverse relations between p14 and p53 expression [158,193]. Immunohistochemical studies in cervical dysplasia and SCCs of the uterine cervix have reported overexpression of both p14 and p16 in HPV-associated cases. In these cases p14 overexpression is attributed to functional inactivation of p53 by the E6 oncoprotein of high-risk HPV, and p16 overexpression is attributed to inactivation of pRb by the E7 oncoprotein of high-risk HPV[166].

In cutaneous carcinogenesis, in which HPV is also implicated, one could hypothesize that p14 and p16 would be overexpressed in HPV associated cases. In addition based on the above one could expect positive associations between p14 and p16 expression levels and inverse relations between p14 and p53 expression.

However, in the present study of a series of 105 KIN lesions and CSCCs, we found the expression of p14 and p53, as well as the expression of p14 and p16, to be independent. With respect to p14/p53 we found absence or presence of both markers more prevalent (56/105 cases) than single positivity (representing inverse relation) for one of the proteins.

Most cases (40%) showed a p14-/p53+ pattern. With respect to p14/p16, a p14-/p16+ pattern was most prevalent (41% of cases).
Apparently, in skin neoplasia the relations between expressions of these tumor suppressors is different from what one would expect based on in vitro data and findings in cervical neoplasia. The reasons for these discrepancies can be threefold.
First, cutaneous carcinogenesis is probably more complex than cervical carcinogenesis, because in skin cancer besides HPV also sun exposition is implicated as a major etiological factor. UV-induced mutations have been found in p53 and INK4a-ARF [19,129,187] and these mutations can affect protein expression.
Secondly, oncogenic properties of HPV types implicated in cutaneous carcinogenesis seem different from those of high-risk HPV types in cervical carcinogenesis. In cervical lesions, p53 is inactivated due to the transforming activities of the E6 viral oncoprotein of the mucosal high-risk HPV types, for instance HPV16 [23]. So far, the knowledge on transforming properties of E6 and E7 of cutaneous HPV types is limited. In contrast to cervical cancer, in which high-risk HPV DNA becomes integrated in the host genome, in NMSCs containing presumed oncogenic HPV types, HPV integration is rare, and it was shown that E6 oncoprotein of, for instance, the cutaneous epidermodysplasia verruciformis-associated HPV38 could not degrade p53 protein [34,121]. Surprisingly, HPV 38 E6/E7-positive cells also did not express p16, in contrast to HPV 16 E6/E7-positive cells, whereas both HPV 38 and HPV 16 E7 had similar biological properties and both were able to inactivate pRb[34].
It is speculated that in skin carcinogenesis E6 mediated degradation of p53 is not as important as in cervical carcinogenesis, since in skin tumors p53 is already often silenced by UV-induced p53 mutation [130].
Finally, protein expression levels do not directly reflect changes on the molecular level. For instance for p53 it is known that many of the mutations in the p53 coding region result in a structurally altered, inactive protein that is more stable than its wild type counterpart, resulting in higher levels of protein detectable by antibody. The high prevalence of p53 overexpression in our series, 77/105 cases (73%), could well reflect cases harboring p53 mutations. However, the reasons for overexpression of p14 and p16 in skin pre (cancer) still have to be established; previously in CSCCs inactivating molecular events (mutation or promotor methylation) were reported to have absent expression of p16 and p14 in 82% of cases [32]. At the moment the molecular events underlying p16 and p14 overexpression remain to be investigated; a previous study on mutation analysis of the CDKN2A gene in skin tumors, including 5 Bowen's diseases (BDs) did not demonstrate mutations in any of the BDs [169]. Nindl et al [141] studied overexpression of p16 and HPV presence in skin tumors, and found HPV DNA in all AKs (n=5), all SCCs (n=7), and all SCCs in situ (n=3) with overexpression of p16 in a part of the lesional cells of all these lesions. In contrast normal skin (n=3), and warts (n=2) despite containing HPV in respectively 66% and 50% lacked p16 overexpression. The HPV types found were mucosal in 6 cases (twice in normal skin, BDs and SCCs each), 4 times HPV type 16; 3 of these cases were p16 positive. In 12 cases EV-associated HPV types were present all showing p16 positivity. They suggest that HPV might be responsible for p16 positivity in skin (pre) cancers and less likely genetic alterations as mutation. However, we conclude that as long as its unknown what enhanced or absent protein expression reflects on the molecular level, it remains difficult to explain relations between expression of markers.
Future studies including cutaneous and EV-associated HPV types with incorporation of molecular data are warranted to clarify the relation between expression of p14, p16, and p53 and HPV infection.
The present study showed that expression pattern for p14/p53 and p14/p16 were conditional independent of immune status and sun exposure. This suggests that skin cancer development

in RTRs and ICIs is comparable with respect to involvement of cell cycle associated proteins, despite differences in immune status and although there is a clear clinical difference in rate of tumor development between both groups.

We could only demonstrate HPV X in 2/108 (1.9%) specimen, and none of the KIN lesions or CSCCs of RTRs and ICIs contained one of the known mucosal HPV types. Due to this low prevalence of mucosal HPV types relations between presence of HPV and expression levels of the three studied tumor suppressors could not be studied. Data on frequencies of mucosal HPV types in skin tumors in literature are conflicting. Previously de Jong-Tieben et al [43] also reported no mucosal HPV types in 96 epithelial skin tumors of RTRs by nested PCR. They only found high frequencies of epidermodysplasia verruciformis (EV) HPV types (15, 19, 20,21, 23, 24, 25 and 38) in 80% of CSCCs and 93% of AKs.

In contrast to our data and those of de Jong-Tieben et al [43], Soler et al [186] reported a high frequency of mucosal HPV types 6/11 with 30 out of 43 benign, premalignant and malignant cutaneous lesions of transplant recipients being HPV positive. Iftner et al and Shamanin et al also both reported presence of mucosal HPV types in NMSC. Iftner et al [89] reported presence of high-risk mucosal HPV types in 18.2% (10/91 cases) of KIN lesions and in 20.9% (9/72 cases) of CSCCs of ICIs. Shamanin et al [173] only found high-risk HPV types in malignant skin tumors of RTRs, with malignant skin tumors of ICIs only containing low-risk mucosal HPV types. We choose for a combined SPF-PCR-LiPA system for detection and genotyping of HPV since this HPV detection test is highly sensitive, specific, and reproducible and has been clinically validated [104,134,165]. Based on this solid technique we dare to conclude that our data do not support a role for mucosal HPV types in cutaneous carcinogenesis.

With respect to p14 expression, in total 42/105 (40%) cases were positive. In LKIN lesions 22% was p14 positive, in HKINs 57%, and in CSCCs 50% of cases were p14 positive. These differences in presence of p14 expression were weakly significant between the three diagnostic groups of LKIN, HKIN and SCC.

So far only Brown et al studied p14 expression in 40 cutaneous SCCs, of which 30 were derived from immunosuppressed patients [32]; they reported p14 expression in 18/40 cases (45%) of CSCCs, which is comparable to our finding of 50% p14-positive carcinomas. p14 staining was not restricted to certain epidermal layers in KIN lesions. This contrasts to already previously reported findings with respect to p16 and p53 staining [18]. The localization of these two markers significantly correlated with KIN grade, with higher KIN grades showing increase in epidermal staining extending from the basal layer to the surface. Therefore our data do not support a role for p14 immunohistochemistry in the diagnosis of epidermal skin tumors.

P14 staining was generally difficult to discern in microscopical slides necessitating high magnification in order to see the weak nucleolar dotted or speckled staining with mostly low number of positive staining cells, in contrast to the much easier to discern and mostly stronger staining for p16 and p53.

In conclusion, based on presented data p14 protein expression does not attribute to the diagnosis of epidermal neoplasia and mucosal HPV types do not play a role in these tumors. Expression of p14 and p53 and of p14 and p16 are independent in KIN lesions and CSCCs and these independencies are not influenced by sun exposure and immune status. The reasons for overexpression of p16 and p14 in especially HKIN lesions and CSCCs need further elucidation, which is also true for the role of non-mucosal HPV types.

2.3

INK4A-ARF and p53 mutations in metastatic cutaneous squamous cell carcinoma

Case report and archival study on the use of Ink4a-ARF and p53 mutation analysis in identification of the corresponding primary tumor

Abstract

So far histopathologic, immunohistochemical and molecular properties of metastatic cutaneous squamous cell carcinomas (CSCCs) are relatively unexplored. In patients with multiple CSCCs, as for instance renal transplant recipients (RTRs), it might prove difficult to identify the primary tumor responsible for metastasis. We report a case of a RTR with multiple CSCCs, one of which metastasized. By using p53 and INK4a-ARF mutation analysis we identified the responsible primary tumor due to an identical mutation in exon 2 of the INK4a-ARF locus. Archival study yielded 14 cases of metastatic CSCC (present case included). In only 8/14 metastases, DNA quality was sufficient to perform PCR reactions. In 7 of /8 metastases either an INK4a-ARF (6 of 8 cases) and /or p53 (3 of 8 cases) mutation was present. In 6 of 7 cases the corresponding primary could be identified by an identical mutation in p53 and/or INK4a-ARF.

In conclusion, molecular analysis using a combination of p53 and INK4a-ARF mutation analysis can identify the corresponding primary skin tumor in case of CSCC metastases in the majority of cases. This is facilitated by the high frequency of these mutations in metastatic CSCC when compared to frequency spectra reported in the literature in primary CSCCs. The major limitation was formed by insufficient DNA quality in archival tissue.

INTRODUCTION

With still improving graft and patient survival in renal transplant recipients (RTRs), nephrologists, dermatologists, and subsequently pathologists, are increasingly confronted with cutaneous complications in this patient group. Of the latter, post-transplantation skin (pre) cancers are the most prevalent. RTRs develop multiple warts, actinic keratoses (AKs), and non-melanoma skin cancers (NMSCs), especially on sun-exposed parts of the body. Of these NMSCs, squamous cell carcinomas (SCCs) by far outnumber basal cell carcinomas (BCCs), in contrast to NMSCs in immunocompetents. Cutaneous SCC (CSCC) is associated with a substantial risk of metastasis with reported rates of metastasis up to 5% [3] in the general population and between 5-10% in transplant patients [50]. Once metastasized, the prognosis is poor in organ transplant recipients [125].

So far, histopathologic, immunohistochemical and molecular properties of metastatic cutaneous SCCs are relatively unexplored [3,23,35,96,108]. Of the first, especially large size (>2 cm in diameter) and depth >4 mm seem associated with metastasis [3,108].

Especially in RTRs, there are often multiple CSCCs and it might prove difficult to identify the primary tumor responsible for the subsequent metastasis. Previous studies in non-skin cancers have already demonstrated the value of the mutational status of p53 in separating or comparing primary and metastatic tumors in the case of multiple primary cancers [48,97].

Molecular data have shown that P53 mutations are the most frequent genetic alteration in primary SCC of the skin with reported incidence of 50% in sporadic SCCs and 43% in SCCs of RTRs [129]. More recently, mutations in the CDKN2A (INK4a-ARF) locus, which maps to chromosome 9p21[163], were reported in primary CSCCs. This locus, by alternative transcripts encodes for two proteins $p16^{INK4a}$ (exons 1α, 2 and 3), and $p14^{ARF}$ (exons 1β and 2). Mutations of $p16^{INK4a}$ have been reported in up to 24% of cases in sporadic primary CSCCs [110,188] and in 33% of CSCCs in xeroderma pigmentosum patients[187].

In this article we want to test the feasibility of using combined INK4a-ARF and p53 mutation analysis in identifying the corresponding primary skin SCC, especially in case of multiple primaries. We illustrate this by means of a case report and an archival study.

Case

A female patient born in 1953 underwent a bilateral nephrectomy when she was 17 years of age (1970). A renal transplantation was performed twice, in 1972 and in 1978 after a chronic vascular rejection of the first transplant.

Immunosuppressive treatment consisted of Prednisone 2dd 5 mg and Azathioprine 1dd 100mg.

Since 1990, 18 years after the first renal transplant, she developed her first skin tumors. In the beginning predominantly basal cell carcinomas, hyperkeratoses and keratoacanthomas. Since 1998 she progressively started to develop AKs, Bowen's disease and SCCs, the latter at a rapidly inclining rate up till now, the end of 2003 (Figure1). In 1996 chemopreventive therapy with acitretin was started in order to inhibit skin tumor development. In July 2002, fine needle aspiration of an enlarged right supraclavicular lymph node demonstrated malignant cells consistent with squamous cell carcinoma metastasis.

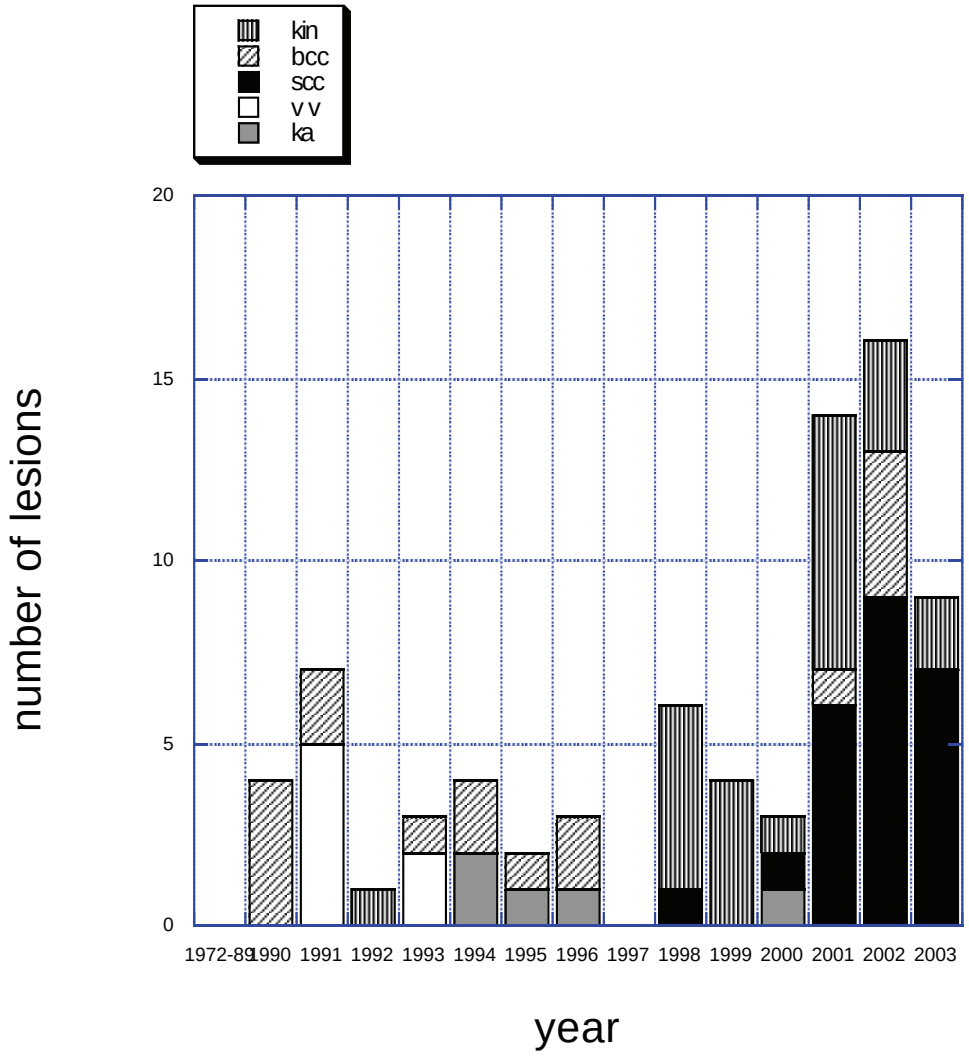

FIGURE 1
Since 1990, 18 years after the first renal transplantation, the patient began to develop skin tumors, first predominantly basal cell carcinomas (bcc), hyperkeratoses, and keratoacanthomas (KA). Since 1998, she developed actinic keratoses, Bowen's disease (taken together as keratinocytic intraepidermal neoplasia, KIN) and squamous cell carcinomas (SCCs), the latter at a rapidly inclining rate up till the end of 2003.
vv=verruca vulgaris.

In August 2002, a right-sided modified neck dissection (containing regions III-VI) was performed. Only in region IV a solitary extranodal metastasis of SCC (Figure 2, A-B), measuring 2cm in diameter, was present with positive surgical margins. The patient received postoperative radiotherapy. Up till now, January 2004, patient is alive without evidence of metastases.

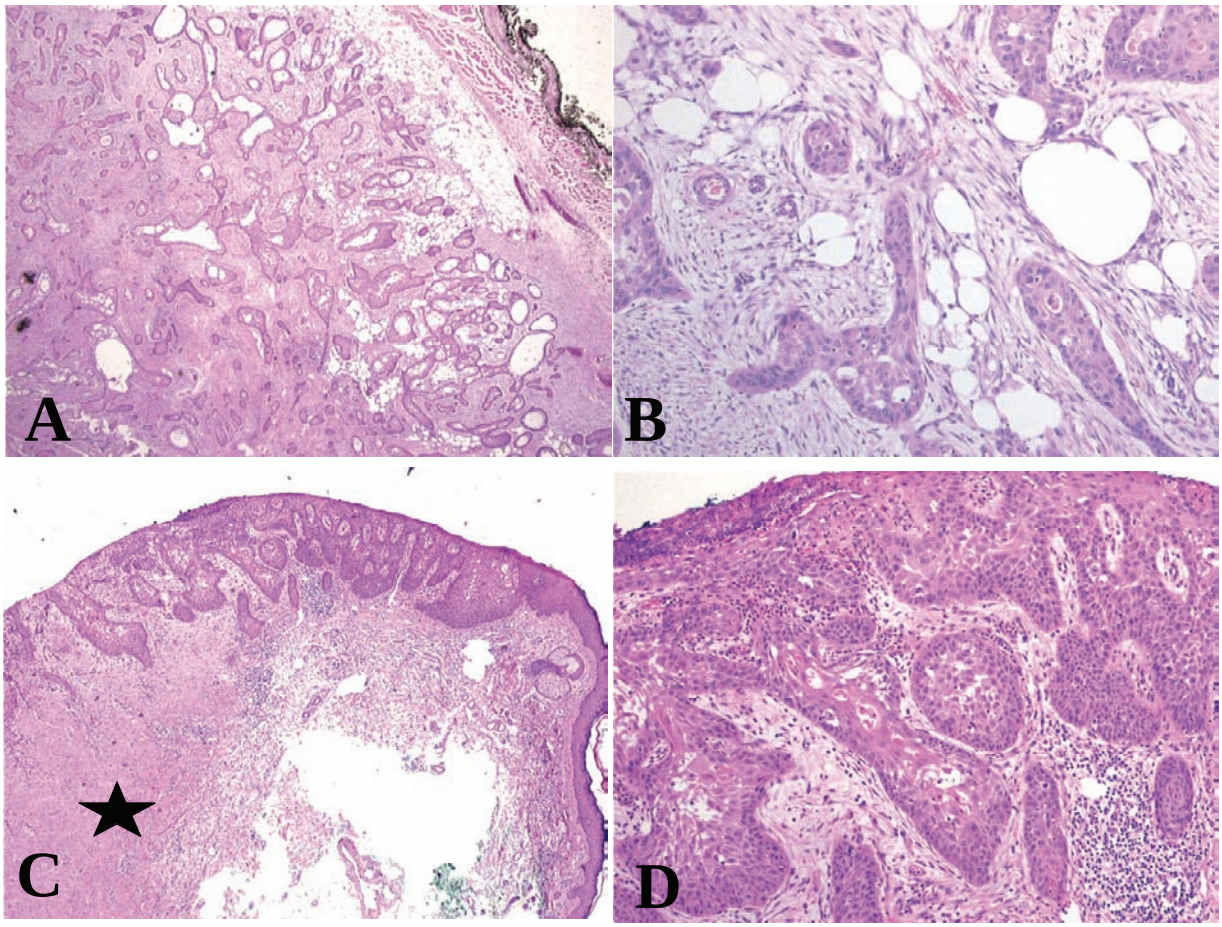

FIGURE 2
A. Right cervical squamous cell carcinoma metastases of the presented case, developing in August 2002, 13 months after the biopsy of the primary tumor located on the right clavicle. No preexistent lymph node could be recognized. In **B** more detailed picture of the tumor.
C. Excision of the primary on the right clavicle, showing a superficial infiltrating moderately differentiated squamous cell carcinoma. In the deeper dermis, scar tissue from the previous biopsy is seen (asterisk). The black line indicates the tumor area which was scraped from the already HE stained slides in order to perform molecular analysis. In **D** more detail of the tumor.

Archival patient selection

In the period between 1985 and the end of 2001, 14 metastases (13 nodal) from 14 different primary cutaneous SCCs from 12 patients, could be retrieved including the above-mentioned patient. In the same time period the number of diagnosed primary CSCCs in our hospital numbered 1345.
In 6 cases patients were renal transplant recipients (RTRs).
The patient characteristics are summarized in Table 1**.**

Table 1.. Patient characteristics.

Patient	Metastatic tumor	Sex	RTR/ICI	Age SCC metastasis	Location candidate primary SCC	Location LNN metastases	Number of NMSCs before first CSCC metastasis
1	I, case	F	Rtr	63	R clavicle	R cervical	12 SCCs
2	D	M	Rtr	53	R hand	R axilla	13 SCCs
3	E	M	Rtr	67	R hand	R axilla	5 SCCs
4	F1	M	Rtr	63	L arm	L axilla	6 SCCs
	F2			63	R temple	R parotic	
5	G	M	Rtr	67	R cheek	R cervical	5 SCCs
6	H	M	Rtr	52	R upper arm	R axilla	NA
7	A1	M	Ici	79	L temple	L resp R	5 SCCs
	A2			80	R ear	cervical and parotic gland	Multiple BCCs
8	B	M	Ici	70	Chin	Cervical	1 KA
9	C	M	Ici	76	R ear	R cervical and parotic gland	0
10	J	M	Ici	44	R neck	R cervical	1
11	K	M	Ici	86	L cheek	L cervical	0
12	L	M	Ici	80	R foot	R inguinal	NA

MATERIALS AND METHODS

For the DNA isolation about 3 sections of 10 μm per 1 cm^2 formalin fixed paraffin-embedded tissue were manually microdissected from unstained sections to obtain a tumor cell percentage of at least 60%, using HE colored sections as a reference. The sections were incubated in P-buffer (50mM Tris pH 8,5, 0.1 M NaCl, 1 mM EDTA, 0.5% Tween-20, 0.5% NP40, 20 mM DTT) for 15 minutes at 90^0C, 16 hours at 60^0C in the presence of 0.2 mg Proteinase K and 5 minutes at 95^0C. Subsequently, DNA was extracted essentially as described by Miller [138]. Depending on the DNA quality as determined in a control PCR, 0.1 to 2 μl of the isolated DNA was used in all PCRs. PCR was performed with AmpliTaq Gold DNA Polymerase (Roche Applied Biosystems) using an optimized $MgCl_2$ concentration (varying from 1 to 2 mM) in a MJ PTC 200 PCR cycler (Biozym). Conditions were 1 min at 92^0C, 1 min at 60^0C , 2 min at 72^0C for 45 (INK4a-ARF) or 40 (p53) cycles with 10 min 94^0C prior to and 10 min 72^0C after cycling.

In each experiment a positive control and a control without template were included to monitor the efficiency of the PCR and the absence of cross-contamination. After purification of the PCR products using a Qiaquick PCR purification kit (Qiagen), sequence analysis was performed using BigDye terminator version 2 of Applied Biosystems on an ABI 3730 automatic sequencer (Applied Biosystems). The amplicons allowed automatic sequence analysis of the complete exons and surrounding splice sites. Since most exons are too large for efficient amplification of formalin fixed DNA, for most exons overlapping PCR products were generated . All primers used in the analysis of p53 contained either an M13 forward or M13 reverse consensus sequence. Sequence analysis of these PCR products was performed using M13 consensus primers. For INK4a-ARF the primers that were used in the PCR were also used in the sequencing procedure. All primers and sizes of the PCR products are listed in Table 2.

The entire open reading frames of INK4a-ARF and exon 5 up to and including exon 8 of TP53 (encoding p53) were analyzed in DNA isolated from the metastases. Mutations were confirmed on an independent PCR product. Alterations that were also present in normal tissue of the patient were considered to be polymorphisms and were excluded from the

study. The analysis of the primary tumors was restricted to the fragments containing the mutations identified in the metastases.

Table 2.. Primers used in the analysis of INK4a-ARF and p53, and the size of their PCR products.

INK4a-ARF (CDKN2A)			
exon	Forward	Reverse	Size PCR product (bp)
1B	TCAGGGAAGGCGGGTGCGCG	GCCGCGGGATGTGAACCAC	244
1B	CGCCGCGAGTGAGGGTTTT	CACCGCGGTTATCTCCTCC	263
1A	GAGAGGGGGAGAGCAGGCAG	GCACCTCCTCTACCCGACC	122
1A	GGAGCAGCATGGAGCCTTC	AGTCGCCCGCCATCCC CTG	177
2	AGCTTCCTTTCCGTCATGC	GCAGCACCACCAGCGTGTC	202
2	AGCCCAACTGCGCCGACCC	CCAGGTCCACGGGCAGACG	146
2	TGGACGTGCGCGATGCCTG	GGAAGCTCTCAGGGTACAAATTC	188
3	CGGTAGGGACGGCAAGAGAG	GAGGGACCTTCGGTGACTGATG	162
p53 (TP53) [1]			
5	TCACTTGTGCCCTGACTT	TCATGTGCTGTGACTGCTTG	179
5	CAGCTGTGGGTTGATTCCAC	GAGGAATCAGAGGCCTGG	196
6	GAGACGACAGGGCTGGTT	GAGACCCCAGTTGCAAAC	234
7	CCAAGGCGCACTGGCCTC	GAG GCAAGCAGAGGCTGG	249
8	CCTTACTGCCTCTTGCTTC	CCCCTTTCTTGCGGAGATTCTC	136
8	TTGAGGTGCGTGTTTGTGCC	TGAATC TGAGGCATAACT	181

1. All forward primers contained the M13 consensus sequence TGT AAA ACG ACG GCC AGT at the 5' end. All reverse primers contained the M13 consensus sequence CAG GAA ACA GCT ATG ACC.

RESULTS

Case

At the moment of the right supraclavicular metastasis, the patient already had a history of 12 cutaneous SCCs; located at the left hand/forearms (8 cases), right clavicle (1 case, once reexcised), left clavicle, and 2 cases on the right forearm. Based on the drainage pattern, the right supraclavicular metastasis was most likely an in transit metastasis of the primary located on the skin of the right clavicle.

Histopathological examination of this tumor was rather unremarkable. In the previous 3 mm biopsy of this tumor performed in July 2001, tumor depth was 2.5 mm and the tumor was limited to the deep dermis. In the excision 1 month later, only a small (-tumor diameter of 8mm) moderately differentiated SCC with ulceration and an infiltrative growth pattern (Fig. 2 C-D) was present. Invasion depth was only 1 mm and the tumor was limited to the upper dermis. The tumor involved the resection margin in this initial excision and was later reexcised (October 2001) with only little residual tumor and free margins this time (of 0.5 mm focally). Simultaneously with the SCC on the right clavicle the patient presented a much larger SCC on the left clavicle with tumor diameter of 2.5 cm and infiltration of 4.5 mm into the subcutaneous fat and this tumor was partially poorly differentiated. Because of the more aggressive histopathological characteristics we decided to include this tumor in the molecular analysis together with the small primary on the right clavicle.

The metastasis contained a mutation in exon 2 of the INK4a-ARF locus involving both p14 (391^393 delCGCinsGA) and p16 (225^227delCGCinsGA). No p53 mutation was detected. In the primary tumor on the right clavicle an identical mutation in the INK4a-ARF locus could be demonstrated. The primary on the left clavicle contained a different mutation in exon 2 of the INK4a-ARF locus involving both p16 (155^178del) and p14 (321^344del).

Archival study

In only 8 of 14 lymph node metastases DNA quality was sufficient to perform PCR reactions. In 7 of 8 metastases, mutations in either INK4a-ARF (6/8 cases) and/or p53 (3/8 cases) were detected (Table 3**)**. Patient and tumor numbering in table 3 corresponds to the numbering in table 1. In 2 of 8 cases combined INK4a-ARF and p53 mutations were present.

In 6 of 7 cases, the primary cutaneous SCC could be identified with certainty due to the presence of an identical mutation in primary and metastasis in either p53 and/or INK4a-ARF (Table 3). This table also contains the available relevant histopathological data and clinical data of all tested cases. In 4 cases the primary SCC was moderately differentiated, in 2 cases poorly differentiated. Diameter of the tumors was between 8mm up to 8 cm. Depth of the tumors varied from only 2.5 mm to deep invasion into the cortical bone. In only 4 cases there was available clinical follow-up with 2 patients being death of disease.

As can be read from the last column in Table 1, in the archival study, akin to the case report, 5 patients (patient nos. 2, 3, 4, 5, and 7) had more than one previously diagnosed primary skin cancer before the metastasis developed. Of these patients only in patient nos. 4, 5 and 7 the DNA quality of the metastatic tumor was sufficient to perform molecular analysis for INK4a-ARF and p53 mutations.

In patient no. 4 the metastasis contained no mutations. Due to absence of p53 and INK4A-ARF mutations in the metastasis and our decision to restrict the analysis of the primary tumors to the fragments containing the mutations identified in the metastasis, none of the diagnosed CSCCs of this patient were tested.

In patient no 5, the metastasis contained p53 and p14 mutations: we tested 2 possible primary tumors but could not demonstrate these mutations in either one of these tumors (Table 3). The metastasis was located in the right cervical lymph node (lnn), and the two tested primaries were located on the right cheek and ear respectively. As can be read from table 1 this patient had 5 previous SCCs: the other 3 tumors were less likely to have caused the right sided cervical lnn metastasis since they were all located on the left side of the face and therefore these tumors were not further tested.

In patient no. 7 the metastasis contained a p16 mutation, which was confirmed in the most likely primary tumor located on the right ear, and hence no other CSCCs of this patient were tested (this patient had 5 previously diagnosed CSCCs, Table 1). Furthermore the other previously diagnosed CSCCs in this patient were all located on the left side of the face, and were less likely to have caused the right parotic lnn metastasis based on lymph drainage.

Table 3. Mutations in p16^{INK4a}, p14^{ARF,} and p53 found in 8/14 tested cases of metastatic CSCCs. In case an identical mutation in the metastases could be confirmed in the candidate primary CSCC histopathological features of the primary skin tumor are listed. Clinical follow-up when available is listed.

Patient/ Tumor	Mutation p16 Exon, codon, base change, AA change, mutation type	Mutation p14 Exon, codon, base change, AA change, mutation type	Mutation p53 Exon, codon, base change, AA change, mutation type	Mutation in candidate primary	Histopathological features of primary	Follow-up
1/I Case	+ Exon 2 225-227del CGCinsGA	+ Exon 2 391-393delCGC insGA	-	P16 and p14 mutations confirmed	Mod.differentiated 8 mm diameter Infiltrative growth Depth: 2.5 mm, limited to dermis. Ulceration.	NT>'96 Alive 2004 without evidence of new metastases
4/F2	-	-	-	P53 no mutation. P16 and p14 not further tested		Reduction immunosuppression and NT '97 Death of lung metastases from cutaneous fibrosarcoma
5/G	-	+ Intron 1B IVS1B+1G>A Inactivation	+ Exon 8 843-844 CC>TT (R282W) UV	- p53 and p14 mutations not found in 2 tested candidate primaries		NT>'96 Died of asthma cardiale with rapidly progressive relapse of cutaneous SCC of the cheek
6/H	+ Exon 1A 143C>T (P48L) UV	-	+ Intron 8 IVS8+1G>A	p53 mutation confirmed. p16 not confirmed: poor quality.	Poorly differentiated 8cm diameter Acantholysis Depth: invasion cortical bone Perineural growth Blood vessel invasion Ulceration and necrosis	NA
7/A2	+ Exon 1A 148C>T (Q50X) UV	-	-	P16 mutation confirmed	Mod.differentiated 11mm diameter Infiltrative growth Invasion in subcutis, Depth 6mm Erosion	NA
8/B	-	-	+ Exon 7 734G>A (G245D)	P53 mutation confirmed	Mod.differentiated >15mm diameter Acantholysis Depth? (only biopsy available) Lymphangitis + Erosion	NA
9/C	+ Exon 1A 76G>T (E26X)	-	-	P16 mutation confirmed	Poorly differentiated >2cm diameter Infiltrative growth Depth? Ulceration	NA
10/J	+ Exon 2 172C>T (R58X) UV	+ Exon 2 338C>T (P113L) UV	-	P16 and p14 mutations confirmed	Mod.differentiated 3 cm diameter Depth; 9 mm, into subcutis	Died of disease in 2002 with extensive cervical lymph node metastases
Total	5/8 cases	3/8 cases	3/8 cases	6/7 cases mutation in primary confirmed		

NA= not available. NT=neotygason treatment (acitretin).

DISCUSSION

The present case of an RTR with multiple CSCCs indeed illustrates that even a very small and histopathological unremarkable SCC can lead to metastasis. Previously, Joseph et al. also demonstrated that in 34 of 695 metastasizing CSCCs, the majority of the primaries were small with 28 tumors measuring 1.5 cm or less[96]. The presented case and archival study of metastatic cutaneous SCCs in our institution, show that mutation analysis of both p53 and INK4a-ARF is very helpful in identifying primaries in case of metastatic CSCC. The only drawback was the frequent insufficient quality of the DNA in archival paraffin embedded material, which led to loss of 6 of14 cases for further molecular analysis. On the other hand, as illustrated by the presented case, even very little tumor tissue can be sufficient in identifying the primary. In this particular case tumor tissue actually was scraped from already cut and HE stained slides.

Seven of 8 tested metastases contained either an INK4a-ARF and/or p53 mutation that subsequently leads to the identification of the primary tumor in 6/7 cases. This high prevalence of INK4a-ARF/p53 mutations in metastatic CSCCs makes this gene combination highly suitable for identification of primary SCCs of the skin.

So far, only a few studies have addressed histopathologic, immunohistochemical and molecular characteristics of metastatic CSCCs. However, in these studies, the supposed primary could not be linked with certainty to the metastasized tumor, due to lack of molecular tools[3,23,35,96,108].

Especially in the group of RTRs, molecular analysis could yield future important information on factors important for metastasis, since in this group SCCs are often multiple and histopathological characteristics are not always indicative of aggressive behavior as illustrated in this study by the presented case.

In the literature, reported frequencies of p16 mutations in sporadic CSCCs vary from 15% (3/21 cases)[110] to 19% (8/42 cases of CSCCs)[187]. P14 mutations in the sporadic CSCCs have a reported frequency of 17% (7/42 cases)[187]. In Xeroderma Pigmentosum (XP) patients, p16 and p14 mutations both have a reported frequency of 33% (6/18 cases) [187]. In these patients, tumors often contained multiple mutations in p14, p16, and p53.

In this archival study, with limited patient numbers, metastatic CSCCs were found to have p16 mutations in 63% (5/8 cases) of all cases. P14 mutations were present in 38% of all cases (3/8 cases).

Thus, the frequency of p16 mutations in metastases we found seems higher than those reported for primary CSCCs in both sporadic and XP-associated SCCs. The metastases contained 50% INK4a-ARF mutations that could be attributed to UV-damage, comparable with XP-associated skin tumors. Larger studies are needed to elucidate a potential role for p16 mutations in predicting metastatic potential of CSCCs.

In the general population p53 mutations in primary SCC of the skin are reported in 19% (4/21 sporadic SCCs)[187] up to 50% (3/6 SCCs)[129]. In XP patients 44% (8/18 cases) of SCCs contained p53 mutations [187]. In RTRs, McGregor reported ten p53 mutations in 9/21 (43%) SCCs of transplant recipients [129], while Bennett only reported p53 mutations in 2/25 SCCs of RTRs [10]. In SCCs of epidermodysplasia verruciformis (EV) patients, 5 SCCs (62.5%) contained p53 mutations [147].

In 3/8 metastases we detected a mutation in p53. This frequency is fairly consistent with reported frequencies for p53 mutations in sporadic primary CSCCs and primary CSCCs in special patients groups. In 1/3 metastasis, p53 mutation could be UV-induced.

In conclusion, the present study shows that molecular analysis of p53 and INK4-ARF mutations can identify the primary CSCC responsible for the metastasis in the majority of cases. This is facilitated by the high frequency of p53 and/or INK4a-ARF mutations we

found in metastatic CSCC, when compared to frequency spectrum reported in the literature in primary CSCCs. The major limitation is formed by insufficient DNA quality in archival tissue.

Chapter 3

RETINOID TREATMENT IN BENIGN AND (PRE) MALIGNANT EPIDERMAL TUMORS OF RTRS

This chapter was based on the following publications :

Retinoids strongly and selectively correlate with keratin K13 and not K19 in cutaneous warts of renal transplant recipients

Willeke A.M. Blokx, M.D[1], Jurgen V.Smit, M.D.[2], Elke M.G.J. de Jong, M.D., Ph.D.[2], Monique M.G.M. Link[1], Peter C.M. van de Kerkhof, M.D., Ph.D.[2] , Dirk J. Ruiter, M.D., Ph.D.[1]

Departments of Pathology[1] and Dermatology[2], University Medical Center St. Radboud, Nijmegen, the Netherlands

Arch Dermatol 2002;138(1):61-5

Acitretin treatment in (pre)malignant skin disorders of renal transplant recipient: histological and immunohistochemical effects

Jurgen V.Smit, M.D.[2], Ruud G.L. de Sévaux, M.D.[3], Willeke A.M. Blokx, M.D[1], Peter C.M. van de Kerkhof, M.D., Ph.D.[2], Andries J.Hoitsma, M.D., Ph.D.[3], Elke M.G.J. de Jong, M.D., Ph.D.[2]

Departments of Pathology[1], of Dermatology[2], and of Nephrology[3], University Medical Center St. Radboud, Nijmegen, the Netherlands[3]

J Am Acad Dermatol 2004;50:189-96

Immunohistochemical effects of temporary cessation of long-term acitretin treatment in keratinocytic intraepidermal neoplasia of renal transplant recipients

Willeke A.M. Blokx, M.D[1], Jurgen V. Smit, M.D.[2], Peter C.M. de Wilde, D.M.D., Ph.D.[1], Peter C.M. van de Kerkhof, M.D., Ph.D.[2], Dirk J. Ruiter, M.D., Ph.D.[1], Elke M.G.J. de Jong, M.D., Ph.D.[2]

Departments of Pathology[1] and Dermatology[2], University Medical Center St. Radboud, Nijmegen, the Netherlands

Arch Dermatol 2003;139:671-73

3.1

Retinoids strongly and selectively correlate with keratin 13 and not keratin 19 expression in cutaneous warts of renal transplant recipients

Abstract

Objective *To compare the expression of keratin (K)13 and K19 in cutaneous warts of renal transplant recipients (RTRs) and immunocompetent individuals (ICIs).*

Design *Retrospective, nonrandomized immunohistochemical study.*

Patients *Specimens form cutaneous warts of RTRs and ICIs were retrieved from the archives of the Department of Pathology, University Medical Center St Radboud, Nijmegen, the Netherlands. Twenty-one warts from RTRs and 21 from ICIs were examined. Nine RTRs (10 specimens) received either systemic acitretin or topical all-trans-retinoic acid, and their effect on both keratins was assessed.*

Main outcome measures *Frequency and expression patterns of K13 and K19 in warts of RTRs vs. ICIs and the effect of retinoids.*

Results *A significantly higher percentage of warts of RTRs expressed K13 compared with warts of ICIs (86% vs. 14%, respectively; P< 0.001). In warts of RTRs, retinoid treatment correlated significantly with a particularly strong, segmental K13 expression pattern, which we termed zebroid. Without use of retinoids, K13 was mostly restricted to suprabasal single cells. Keratin 19 was absent in all warts of both patient groups.*

Conclusions *Retinoids strongly correlate with K13 in a characteristic zebroid pattern in warts of RTRs, making K13 a sensitive marker for retinoid bioactivity in skin (lesions) of RTRs.*

In non-retinoid-treated RTRs, K13 is also frequently found in warts, but without the dramatic zebroid pattern noted with retinoid-treated warts.

INTRODUCTION

Epithelial keratins comprise a heterogeneous group of acidic (type I) and neutral-to-basic (type II) proteins. As a general rule, they are coexpressed in specific pairings, each pair consisting of a type I and type II keratin. For instance, in normal adult skin, keratin pairs K5/K14 and K1/K10 predominate in the basal layer and the suprabasal compartment, respectively [185].

The type I keratins K13 and K19 are usually expressed separately in adult epithelia. Combined expression occurs only in fetal skin. Both keratins are thought to be absent in normal skin of adults except at certain body sites, such as the penile foreskin, which still contains K13. Furthermore, K13 is abundantly present in adults in internal stratified epithelia and associated (with terminal differentiation) with suprabasal expression [114,139,185,200]. Expression of K19 in adults is found in simple epithelia, such as most glandular epithelia.

In the past few years several murine and *in vitro* studies have demonstrated that vitamin A and its derivates (retinoids) are important regulators of epidermal differentiation and affect keratin gene expression. In cultured keratinocytes, the induction of an embryonic type of differentiation by retinoids with reinduction of K13 and K19 expression has been well documented [106,107]. In vivo topical application of retinoids on photo-aged human skin also showed induction of K13 [162].

Although retinoids are used as chemopreventive agents for inhibiting skin cancer in renal transplant recipients (RTRs)[9,217], to the best of our knowledge there are no studies regarding the effects of retinoids on keratin expression in the skin or skin lesions of these patients.

Renal transplant recipients develop multiple warts and skin neoplasms. Immunosuppressive treatment, sun exposure, and viral infection with human papillomavirus are all implicated in the etiology of cutaneous tumorigenesis in RTRs[6,30,149,171,210]. The frequent simultaneous occurrence of warts and skin cancers in RTRs led us to the assumption that the verrucae or warts in RTRs might be more prone to become malignant than equivalent lesions in healthy immunocompetent individuals (ICIs). The existence of high- and low-risk papillomas was previously shown in mice models of cutaneous carcinogenesis [218]. Interestingly, these high-risk papillomas expressed K13, which was absent in low-risk papillomas.

The present study shows that RTR-associated warts in contrast to warts in normal ICIs indeed show pronounced K13 expression. This suggests that altered keratin expression may reflect an important molecular event inherent in the malignant degeneration of warts in RTRs. Furthermore, this K13 expression in warts of RTRs strongly correlates with retinoid therapy, but, in contrast to findings in animal studies and in cultured human keratinocytes [107], we could not demonstrate an effect of retinoids on K19 expression in these patients. Retinoid-related K13 expression in epithelial skin lesion of RTRs displays a highly characteristic pattern, which we termed *zebroid*, making K13 a useful marker for evaluating the effect of retinoid treatment in these patients.

MATERIALS AND METHODS

Tissues

For this retrospective immunohistochemical study we retrieved formalin fixed and paraffin embedded skin excisions of warts from renal transplant recipients (21 excisions, 18 patients, average age 50.8 years, mean duration of immunosuppression 16.4 years) and from normal immunocompetent individuals (21 excisions, 19 patients, average age 33.3 years) out of our archival material at the department of Pathology, University Medical Center Nijmegen St. Radboud, the Netherlands.

Retinoid treatment

Retinoids were only used by renal transplant recipients. Of the 18 patients with warts, 9 patients (10 excisions) received retinoid treatment . In 3 patients (4 excisions) topical all-trans-retinoic acid (concentration 0.025-0.05 %) was used and 6 patients received systemic acitretin (dose at time of biopsy, 10-35 mg).

Patients taking systemic acitretin participated in a clinical trial , unrelated to the present study, studying the effects of systemic retinoid on cutaneous carcinogenesis in RTRs. Inclusion criteria for this trial were either the presence of at least 1 squamous cell carcinoma (SCC) in the patient's history or the presence of 10 or more actinic keratoses, with at least 1 confirmed histologically. Initially, most patients taking acitretin started with 30-35 mg/d, a dosage that prevented SCCs in RTRs in an earlier study[9]. However, in a relatively large number of patients, doses of acitretin had to be lowered because of mucocutaneous adverse effects (peeling of palms and soles and/or cheilitis).

Retinoid- and non-retinoid-treated patients showed no obvious differences with respect to duration, dosage, and type of immunosuppression, all factors implied in the etiology of skin cancers in immunosuppressed patients (data not shown).

Histopathology

Histological examination of all studied lesions was revised according to World Health Organization definitions of verrucae [84]. The verrucae consisted predominantly of common warts or verrucae vulgares, with a smaller group of verrucae plana, especially in the ICIs, usually located on hands and feet. Condyloma acuminata or anogenital warts were not included.

Immunohistochemical analysis

Immunohistochemical analysis was performed on all specimens by using standard avidin-biotin-peroxidase complex system with either diaminobenzidine (DAB) and/or 3-amino-9-ethylcarbazole (AEC) as the chromogens. In brief, 4-μm-thick paraffin sections were deparaffinized, hydrated and washed in buffered phosphate.

For K13 staining, sections were cooked in buffered citrate (10mM, pH 6.0) in a microwave oven 2 times for 5 minutes each (600 Watt). After a cooling down period of (at least) 20 minutes and preincubation with 20% normal horse serum for 15 minutes, the sections were incubated with undiluted primary antibody overnight at 4˚C. We used two mouse monoclonal primary antibodies, 1C7 (Immunoglobulin G2a) and 2D7 (Immunoglobulin G2b), both recognizing K13 [199]. As a positive control, normal esophageal tissue was used. After incubation with primary antibodies, sections were incubated for 30 minutes with biotinylated horse anti-mouse (1: 200 dilution; Vector Laboratories, Burlingame, Calif), followed by incubation for 45 minutes with avidin-biotin complex (1:50 dilution; Vector Laboratories). For development with 3-amino-9-ethylcarbazole, avidin-biotin complex concentrations were doubled.

For K19 immunohistochemical analysis, (mouse) monoclonal antibody RCK 108 (Biogenex, San Ramon, Calif/DAKO) was used. Besides different pretreatment (0.1% pronase for 10 minutes),the same procedure as for K13 was followed. Eccrine ducts and sweat glands served as positive internal controls.

Sections were counterstained with Mayer hematoxylin for 2 minutes.

Immunoreactivity was scored as negative, slightly positive in a suprabasal single-cell pattern (< 10% of lesional keratinocytes positive), or strongly positive in a suprabasal segmental columns pattern (zebroid pattern).

Scoring was performed without knowledge of patient history and use of retinoid therapy.

The Pearson χ^2 test was used for all statistical analyses, and significance was set at $P<.05$.

RESULTS

General aspects of immunostaining for K13 and K19

In all cases, immunostaining for K13 was restricted to the cytoplasm. Slight variations in staining intensity were observed in lesional skin comparing antibodies 1C7 and 2D7 with overall stronger staining with the 2D7 antibody. Principally, however, the staining pattern of lesional skin with both antibodies was identical. Normal esophagus was used as a positive control and showed strong diffuse suprabasal staining.

Keratin 19 immunostaining was also localized in the cytoplasm. Eccrine ducts and sweat glands, serving as internal controls, showed marked positivity.

Expression of K13 and K19 in warts of RTRs vs. ICIs and effects of retinoid treatment

There was a significant difference in K13 expression between warts of RTRs and warts of ICIs ($P < .001$). A high percentage of warts of RTRs (86%, 18 cases) showed K13 expression, whereas in benign warts of ICIs almost all lesions were negative except for 3 (14%) of 21 with suprabasal single-cell positivity (Table 1 and Figure 1).

Table 1. K13-positive and K13-negative warts in RTRs and ICIs*.

	Warts of RTRs 21 lesions	Warts of ICIs 21 lesions
K13 positive	18 (86%)	3 (14%)
K13 negative	3 (14%)	18 (86%)

*K13 indicates keratin 13; RTRs, renal transplant recipients; ICIs, immunocompetent individuals.

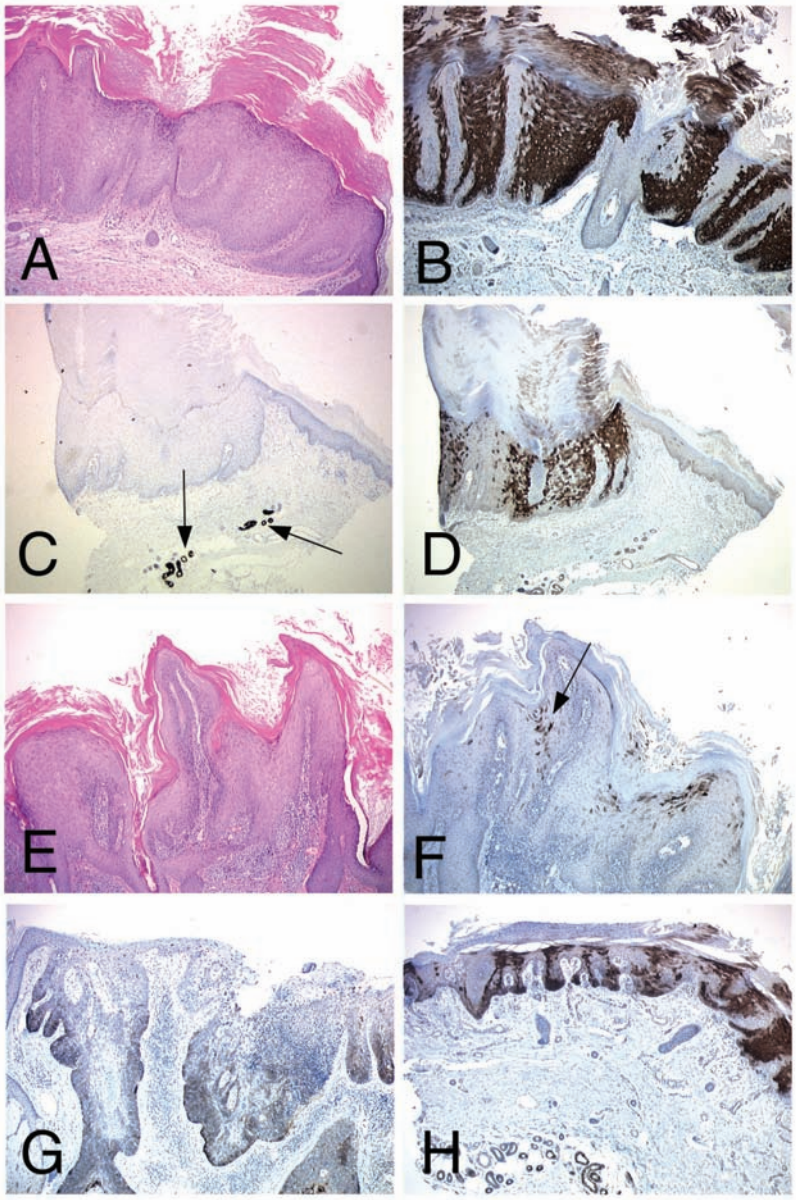

FIGURE 1

A, Hematoxylin-eosin staining of a wart from a retinoid-treated renal transplant recipient (RTR)(original magnification x40). **B**, Immunohistochemical analysis of a wart from a retinoid-treated RTR for keratin (K)13 (monoclonal antibody 2D7, with diaminobenzidine as the chromogen) showing the particularly strong 3+ positive zebroid pattern of alternating suprabasal columns of K13-positive and K13-negative keratinocytes. This zebroid pattern was significantly correlated to retinoid treatment in RTRs (original magnification x40). **C and D**, Wart of a retinoid-treated RTR showing uncoupled regulation of K13 and K19 expression by retinoids, with strong zebroid pattern K13 positivity (D), whereas K19 expression is absent (C). The Arrows (C) point to K19-positive sweat glands, which serve as an internal control (original magnification x40). **E**, Hematoxylin-eosin staining of a wart from an immunocompetent individual (ICI). **F,** Immunohistochemical analysis of a wart from an ICI for K13 (2D7, diaminobenzidine) showing suprabasal single-cell positivity (arrow). This pattern of K13 expression was also typical of warts from non-retinoid-treated RTRs (original magnification x40). **G**, In situ squamous cell carcinoma (ISSCC) of an RTR showing K13 (2D7) expression partially centered around hair follicle structures (original magnification x40). This staining pattern differs considerably from the retinoid therapy related zebroid K13 pattern.

H, Perilesional skin in specimen from an ISSCC of an RTR receiving retinoid treatment. Immunohistochemical analysis of K13 (2D7) shows the same zebroid pattern as in lesional skin of most warts of retinoid-treated RTRs (original magnification x40x).

This statistical difference in K13 positivity remained when we excluded retinoid treated patients; 82 % K13 positivity in non-retinoid-treated RTRs vs. 14% in the controls ($P<.001$). Besides number of positive specimens, the proportion of positive lesional cells also differed and was more pronounced in warts of RTRs: the 3 positive warts of ICIs showed only suprabasal single-cell positivity (Figure 2) ; in RTRs, half of the 18 positive warts also showed this single suprabasal cell positivity, whereas the other half showed strong positive staining in a remarkable pattern of segmental positive suprabasal full epithelium thickness columns (zebroid pattern)(Figure 1B, D and Figure 2). This particular pattern was not linked to eccrine ducts or hair follicle structures; the latter actually seemed to be spared.

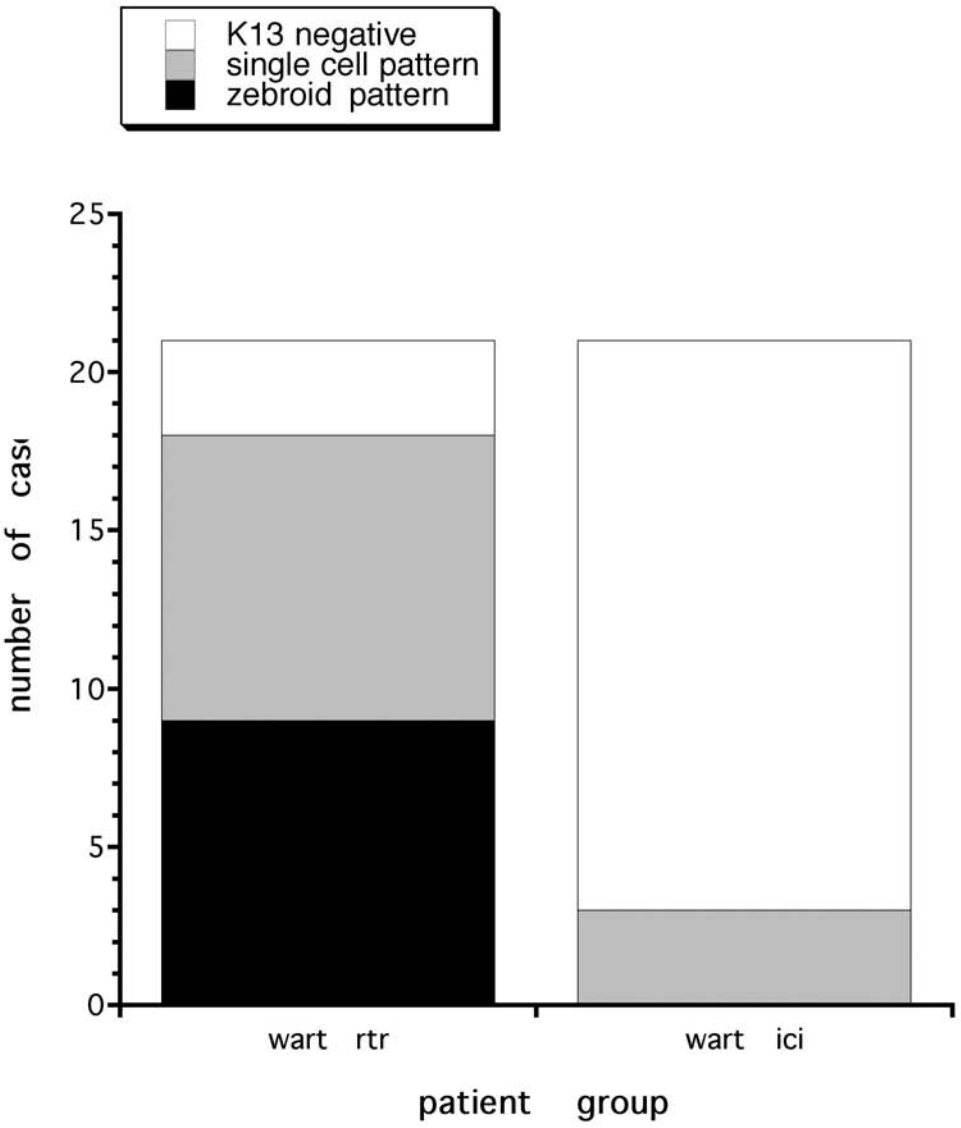

FIGURE 2
Keratin 13 expression patterns in warts of renal transplant recipients (RTRs) vs. immunocompetent individuals (ICIs).

This zebroid pattern correlated with retinoid treatment (topical and systemic) when comparing retinoid-treated RTRs (warts and in situ SCCs) with non-retinoid-treated RTRs (Table 2)(P<.001). Only 1 patient without (anamnestically traceable) retinoid treatment exhibited the same K13 expression pattern.

Table 2 Strong Keratin 13 (K13) positivity (zebroid pattern) and nonzebroid K13 positivity vs. retinoid therapy in renal transplant recipients

	Retinoid treated warts (No., %) n=10	Non-retinoid treated warts (No., %) n=11
K13 zebroid pattern	9 (90%)	1 (9%)
K13 nonzebroid pattern	1 (10%)	10 (91%)

Most warts were superficially excised with no perilesional skin available. In 1 retinoid-treated RTR, the perilesional skin showed K13 positivity comparable with the previously described zebroid pattern.
All warts in both groups were negative for K19, with use of retinoids having no demonstrable effect on K19 expression (Figure 1 C-D).

COMMENT

Data from numerous animal and in vitro studies [55,79,106,107,202] with cultured human keratinocytes have indicated that retinoids influence epidermal proliferation and differentiation. Retinoids repressed expression of differentiation-specific keratins (K1/K10) and strikingly reinduced K13 and K19 expression, two keratins that are only coexpressed in fetal skin but not normally present in epidermis of adults[139,200]. In contrast to these findings of coupled K13 and K19 induction by retinoids, Agarwal was the first to report uncoupled regulation of K13 and K19 expression in a human squamous cell carcinoma cell line[1].
Interestingly, our in vivo data with immunohistochemical evaluation of K13 and K19 expression in warts of RTRs versus ICIs, showed that retinoids used as a chemoprotective agent in the transplant recipients for preventing skin cancer, also only strongly relate to K13 and not K19 expression. Our finding of retinoid-related uncoupled K13 and K19 expression in these patients could be threefold. First, the retinoid concentration in our patients, could be sufficient to enhance K13 expression but not K19 expression. Earlier findings in human epidermal cultures indeed showed stronger induction of K13 than of K19 by retinoids with already a marked increase of K13 in response to very low levels of retinoids, while for K19 induction a higher threshold retinoid concentration was necessary[106]. Although in a considerable number of our patients on systemic retinoids dosages had to be lowered in the course of treatment because of severe mucocutaneous side effects, also in patients still receiving the higher dosages of acitretin no induction of K19 was found. Second, response of keratinocytes to retinoids in vitro might not be representative for the response in vivo and retinoids in humans in vivo might not induce an embryonic type of differentiation and only selectively induce K13. In the only previously performed in vivo study on effects of retinoids, in photo-aged skin, only K13 and not K19 expression was studied[162]. Finally, this differentiation towards “esophageal-type” of epithelium in contrast to so-called “embryonic-type” of differentiation found in animals and in vitro, could be specific for skin and skin lesions in RTRs : earlier studies already showed that effects of retinoids differed in normal keratinocytes when compared to diseased keratinocytes[202].

Retinoid treatment significantly correlated with a specific pattern of K13 expression in skin lesions of RTRs. This pattern, which we termed *zebroid* because of alternating suprabasal columns of K-13 positive and K13-negative keratinocytes, is suggestive of a genetic mosaicism, reflecting clonal expansion of genetically altered stem cells. In warts and slightly dysplastic skin of RTRs, segments of epidermis may contain keratinocytes with a genetic abnormality making them more susceptible to inductive actions of retinoids. Future studies might unravel the underlying genes that are involved in this process and whether, for instance, transforming (transforming types of) human papillomavirus might play a role[152].

Interpretation of the biological impact of K13 expression in retinoid- and non-retinoid-treated warts of RTRs could be 2-fold. The first interpretation relates the presence of K13 in skin to differentiation, in parallel to internal squamous epithelia, in which K13 is restricted to the suprabasal epithelial compartment and is associated with differentiation. Retinoids, by inducing K13 expression or directing keratinocytes towards (esophageal) differentiation, might be chemopreventive by "freezing" cells in this differentiated state and preventing them from (further) dedifferentiating. Results of previous studies [57,106,189] of retinoid effects on epidermal keratinocytes have shown that in response to retinoid treatment, higher molecular weight keratins, typically encountered in squamous epithelia disappear, and synthesis of two new low molecular-weight keratins, a 40 – and 52-kD keratin, corresponding to K19 and K13 respectively , is enhanced. In the absence of vitamin A , the opposite occurs, with enhanced terminal *epidermal type* of differentiation [197] . Retinoid-induced *esophageal-type* differentiation could provide an explanation for the cosmetic improvement of lesional skin in these RTRs, who often had multiple hyperkeratoses before treatment: esophageal epithelium is, in contrast to the keratinizing epidermis, a nonkeratinizing squamous epithelium. By inducing non-keratinizing differentiation, retinoids could lower the number of hyperkeratotic skin lesions. As an adverse effect, in normal skin the diminished cutaneous keratinization caused by retinoids leads to desquamation of palms and soles which usually show the most prominent keratinization. On the lips, the outer cutaneous side becomes more vulnerable because of differentiation towards wet epithelium, leading to cheilitis, another known adverse effect of acitretin treatment also present in our patients [9,80,217].

The second interpretation relates the presence of K13 to a more dedifferentiated and potentially malignant phenotype. Regarding cutaneous carcinogenesis, malignant transformation is heralded by a switch from production of high-molecular-weight keratins normally present in adult skin (K1/K10) to low-molecular-weight keratins also characteristic of fetal skin and simple epithelia (e.g. K8/K18 and K19)[185]. Presence of K13, a low-molecular-weight embryonic keratin, would fit within this concept. It is tempting to attribute relevance to the high frequency of K13 in warts of RTRs and to speculate that it may be related to the higher susceptibility of warts in these patients to become malignant. This would be analogous to mouse models on skin carcinogenesis in which aberrant K13 expression is a consistent finding in chemically and v-Ha-ras-induced papillomas and squamous cell carcinomas[142,194,218]. In in situ SCC of RTRs and ICIs we indeed found frequent K13 expression in 75% and 45% of lesions, respectively (20 in situ SCC tested within each group, data not shown), which is in concert with this second hypothesis. The pattern of K13 expression in in situ SCCs of both groups was different from the retinoid therapy-related K13 expression (zebroid pattern, compare Figure 1 B, G). Only 4 RTRs with in situ SCCs used retinoids, and in these patients the retinoid therapy-related zebroid K13 pattern was most pronounced or only present in perilesional, slightly dysplastic skin (Figure 1H).

When this latter interpretation would be applicable to retinoid-related K13 expression, use of retinoids might actually be dangerous for these patients. This is contradicted by studies of the long term safety of retinoid therapy, since no increased incidence of skin cancer is

reported[196]. Studies of skin cancer chemoprophylaxis with retinoids in RTRs actually showed reduction in the skin cancer incidence[9].
In conclusion, this retrospective in vivo study of embryonic keratin expression in warts of RTRs, shows that retinoids strongly relate to K13 but not K19 expression. By keeping keratinocytes in this esophageal-type differentiation retinoids might act chemo preventively. Retinoids correlate with a highly distinctive and strong K13 expression, which we termed zebroid, making K13 a useful marker for evaluating retinoid treatment in these patients. The alternating zebroid K13 pattern is suggestive of an underlying genetic mosaicism. Even in the *absence* of retinoids, a significant higher percentage of K13 positivity is found in warts of RTRs compared with warts of ICIs. Future prospective, randomized and well controlled studies need to establish the relevance of this finding and whether K13, in analogue to mouse models on skin carcinogenesis, might become a predictive marker for malignant progression.

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3.2

Acitretin treatment in (pre)malignant skin disorders of renal transplant recipients: histological and immunohistochemical effects

Abstract

Background: *The incidence of (pre)malignant skin lesions after renal transplantation is high. Acitretin treatment appears to decrease the number of new squamous cell carcinomas and ameliorates the aspect and reduces the number of actinic keratoses. However, no histological and immunohistochemical studies have been performed to further substantiate these observations.*

Methods*: In 33 renal transplant recipients, biopsies were taken before and after 3 months of treatment with acitretin in doses up to 0.4 mg/kg/day. Histological and immunohistochemical parameters for dysplasia, epidermal thickness, proliferation, differentiation, apoptosis, and dermal inflammation were analyzed.*

Results*: Following acitretin treatment, a significant reduction in epidermal thickness (P= .002) and a significant increase in normal differentiation parameter K10 (P= .02) was observed. Epidermal proliferation did not change, nor did apoptosis, inflammation, keratinocytic epidermal neoplasia score, or transglutaminase staining. At baseline in 8 actinic keratoses a single cell expression pattern of K13 and/or K19 was found. This was associated with high levels of parameters indicative of high-risk lesions (P< .05). After acitretin treatment, an increase in K13 (P= .006) and K19 (P= .05) was found, together with a change in expression towards a focal or band-like staining pattern.*

Conclusion: *acitretin improves the aspect of actinic keratoses via alteration of keratinization, resulting in peeling of the stratum corneum. No significant change in proliferation was found, which may explain for the rapid recurrence of actinic keratoses seen after cessation of acitretin treatment.*

INTRODUCTION

In renal transplant recipients (RTRs) receiving long-term immunosuppressive treatment, an increased incidence of several benign wart-like lesions and (pre)malignant conditions has been found. The most frequently occurring (pre)malignant lesions are actinic keratosis (AK), Bowen's disease, squamous cell carcinoma (SCC), keratoacanthoma, basal cell carcinoma, and porokeratosis [39,50]. Major etiologic factors include previous sun exposure, the duration of immunosuppressive therapy, and human papillomavirus (HPV) infections [50,175]. AKs are the lesions most frequently seen in this population and have been reported in up to 38% of these patients after 5 years follow-up, and even increase thereafter [6]. The percentage of AKs that will convert into a SCC within one year varies between 0.25% and 16% depending on the number, sort and duration of risk factors present, especially the use of immunosuppressive drugs [76].

AKs in RTRs usually are multiple and behave more aggressive when compared to identical lesions in non-immunocompromised individuals [208]. Treatment modalities like surgical excision, cryotherapy, or topical fluorouracil, are often difficult to perform in view of the multiple localizations and involvement of large body areas. Therefore, a systemic treatment preventing malignant transformation is urgently needed.

Data obtained from 3 studies in RTRs where AKs were treated with systemic etretinate or its biologically active metabolite acitretin showed a significant decrease in the number and an improved aspect of the AKs [9,73,102] . In 5 studies it was mentioned that these systemic retinoids also decreased the number of SCCs [9,73,102,132,180] . However, only one of these studies was randomized and placebo-controlled [9]. After cessation of systemic retinoid treatment a recurrence in the number and aspect of AKs [9,102] and in SCCs was frequently seen [9,73,102,132,180,208].

Ideally, the criteria for improvement of AKs comprise both changes in clinical appearance and changes in relevant histopathologic and immunohistochemic features of the preneoplastic process. Clinical improvement without improvement of dysplastic and proliferative characteristics may mask these premalignant keratoses and may mislead both patient and physician. Apart from data on Langerhans cells [72,160], no histological and immunohistochemic studies investigating the effect of systemic retinoids on AKs in RTRs have been performed to strengthen or contradict the clinical impression.

It is known that malignant transformation is represented by dedifferentiation, which is associated with a switch in synthesis of high molecular weight proteins, such as keratin 1 (K1) and keratin 10 (K10), towards low molecular weight proteins, such as keratin 13 (K13) and keratin 19 (K19) [185]. In normal human epidermis, these keratins are absent [139], but they can be expressed in SCCs [209]. No information is available on the expression of these low-molecular weight molecules in AKs.

Therefore, in the present study we evaluated parameters for dysplasia, epidermal thickness, dermal infiltrate, epidermal proliferation, epidermal differentiation, and apoptosis, as well as parameters for dedifferentiation and retinoid-induced keratinization, the low molecular weight proteins K13 and K19 in AKs in RTRs.

MATERIALS AND METHODS

Study design

This study was conducted in adult RTRs with a stable graft function and a stable dose of immunosuppressive therapy. Patients initiated treatment with acitretin 0.4 mg/kg/day, unless it was known from previous acitretin treatment that this dose was intolerable because of side effects. In that case, the highest dose still tolerable by the patients was applied. The duration of treatment was 12 weeks. After this 12-week study period, patients remained on acitretin therapy for another 9 months for further clinical evaluation.

Patients should have a history of at least one SCC of the skin and ≥10 AKs or ≥20 AKs if no previous SCC had occurred. Exclusion criteria were: excessive alcohol intake, the use of anti-epileptic drugs, nephrotic syndrome, hypercholesterolemia (>9 mmol/l), hypertriglyceridemia (>10 mmol/l), elevated transaminase levels (ALT and/or AST more than twice the upper limit of normal) and pregnancy or pregnancy-wish. The washout period for systemic retinoids was 3 months. Topical retinoid treatment had to be discontinued at least 4 weeks before study enrollment to ensure an adequate epidermal turnover (in normal skin 2-3 weeks; in hyperproliferative disorders < 1 week [7]) with respect to potential previous retinoid-associated effects by these agents. Medical Ethics Committee approval was obtained and all patients gave written informed consent prior to study enrollment.

Biopsies and staining procedures

Punch biopsy specimens of 4mm were taken under local anesthesia with xylocaine/1% adrenaline at baseline and after 3 months treatment. Biopsy specimens were taken from AKs that were clinically identical at baseline and had the typical erythematous, scaling and mostly elevated aspect and from the same body region. Suspicious lesions that were present at baseline or that newly formed during the study, were biopsied for diagnosis or, if necessary, surgically removed.

Biopsy specimens were analyzed using standard histological techniques. The following histological parameters were assessed: thickness of the lesions, total dermal infiltrate score (TDI), and keratinocytic epidermal neoplasia score (KIN) [38].

The following immunohistochemical stainings were performed, using the avidin-biotin-complex (ABC) staining method and indirect immunoperoxidase techniques: Ki-67 (MIB-1, epidermal proliferation), p53 (DO-7, apoptosis association), K10 (DE-K10, normal keratinization), K13 and K19 (1C7 and RCK108 respectively, retinoid-associated keratinization), K16 (LL025, hyperproliferation-associated keratinization) and transglutaminase (BT621, terminal differentiation). Esophageal tissue, eccrine ducts, and sweat glands served as positive controls for K13 and 19 staining. Further details on the immunohistochemical markers are provided in Table I.

Table 1. Antibodies used in the study.

antibody	Specificity	Marker for	Concentration	Source
MIB-1	Ki-67	Epidermal proliferation	1:50	Immunotech, Marseilles, France
DE-K10	K10	Normal keratinization	1:100	Monosan, Uden, Netherlands
1C7	K13	Retinoid-induced keratinization	1:10	Monosan, Uden, Netherlands
LL-025	K16	Hyperproliferation Associated Keratinization	1:10	Novocastra lab. Ltd, Newcastle Upon Tyne, UK
RCK108	K19	Retinoid-induced keratinization	1:50	Monosan, Uden, Netherlands
BT621	Transglutaminase	Terminal differentiation	1:10	Biomedical Technologies Inc., Stoughton, Mass.
DO-7	P53	Apoptosis	1:200	Dako, Glostrup, Denmark

Histological and immunohistochemical scoring

With respect to histological analysis, epidermal thickness (mm) was measured in the center of the AKs, and additionally assessed by counting the number of cell layers. TDI scores were performed by using a similar semi-quantitative score on a 5-point scale: 0 = no infiltrate, 1 = minimal infiltrate, 2 = moderate infiltrate, 3 = moderate-pronounced infiltrate, and 4 = pronounced infiltrate. KIN scores were assessed according to Cockerell's criteria for keratinocytic epidermal neoplasia[38]. In brief, lesions were graded based on the degree and extent of dysplasia on a 4-point scale.

Immunohistochemical scoring was based on methods previously published[106] and occurred along the complete length of the slides, but only in the sections that showed typical histological signs of AKs. Transglutaminase scores were assessed in the center of the AKs as the ratio of BT621-positive epidermal cell layers divided by the total number of epidermal cell layers. Ki-67 and p53-positive keratinocytes (nuclei) were counted per millimeter length of section. For scoring of keratinization parameters K10, K13, and K16, the following semi-quantitative scale was used: 0 = no staining, 1 = sporadic staining (single cell expression), 2 = minimal staining, 3 = moderate staining, 4 = moderate-pronounced staining, 5 = pronounced staining, 6 = whole epidermis stained.

All histological and immunohistochemical scoring occurred under blinded conditions. Demographic and dosage-related results are expressed as means ± standard deviation (SD); histological and immunohistochemical results are expressed as means ± standard error of the mean (SEM) unless stated otherwise.

Statistical analysis

For the assessment of statistical significant differences before and after 12 weeks of treatment with respect to the histological and immunohistochemical parameters, a Wilcoxon matched pairs test was used. Assessment of statistical significant correlations between parameters was performed by calculating Spearman's rank correlation. Differences between K13 and/or K19 responders versus non-responders were analyzed by using the Mann-Whitney *U* test. A *P* value of $< .05$ was considered statistically significant. Calculations were performed using Statistica version 9.0 (StatSoft Inc, Tulsa, Okla).

RESULTS

Demographics

Demographic data are summarized in table 2. Thirty-three patients (15 men and 18 women) were included in this study. Most patients also participated in a randomized study focusing on the clinical effects of acitretin on AKs (de Sévaux et al, submitted for publication). The mean age was 53.6 ± 9.7 years. The follow-up period after renal transplantation was 16.4 ± 6.7 years. Twenty-nine patients had a history of one or more SCCs, with a mean of 2.6 ± 2.8 SCCs per patient (excluding keratoacanthomas). Maintenance immunosuppressive treatment consisted of prednisone plus azathioprine (25 patients), cyclosporine plus prednisone (6 patients), mycophenolate mofetil plus prednisone (1 patient), and cyclosporin plus azathioprine (1 patient).

Table 2. Demographic data

Age (y)*	53.6 ± 9.7
Sex (male : female)	15 : 18
Years of immunosuppression*	16.4 ± 6.7
Maintenance immunosuppression	
Azathioprine / prednisone	25
Cyclosporine / prednisone	6
Mycophenolate mofetil / prednisone	1
Cyclosporine / azathioprine	1
Number of previous SCCs (No./patient)	
All previous SCCs	2.6 ± 2.8
During year before study	1.0 ± 1.3
Previous systemic retinoid use	
Within 1 yr before study	0
More than 1 yr before study	3
Previous local retinoid treatment	7

* Values are given as mean ± SD

Dosage

Except for two patients who started with a lower dose (0.2mg/kg/d) due to previous intolerability of higher acitretin doses, the remaining 31 patients initiated treatment with acitretin 0.4 mg/kg/d. Most of these patients could not tolerate the starting dose of 0.4 mg/kg/d because of mucocutaneous side effects (mostly cheilitis, intensive peeling, and eye irritation/conjunctivitis). Therefore, all patients received the maximum dose of acitretin that

was still tolerated. The starting dose was 39.8 ± 8.6 mg/day (0.38 ± 0.07 mg/kg/d). At the end of the 12-week treatment period, the mean dose had been reduced to 20.2 ± 9.6 mg/d (0.26 ± 0.13 mg/kg/d).

Histologic results

Biopsy specimens taken at baseline and after 12 weeks of treatment with acitretin were available in 32 out of 33 patients. One patient withdrew from the study because of severe mucocutaneous side effects after 8 weeks of treatment. In 28 baseline samples a typical actinic keratosis was found; in 2 samples, only hyperkeratosis without dysplasia was seen; in 1, actinic keratosis had already progressed to a SCC; and 1 hyperkeratotic lesion appeared to be a common wart. One biopsy was not intact and was therefore excluded from evaluation. Therefore, 27 pairs of biopsies taken before and after acitretin treatment could be used for histological and immunohistochemical analysis.

On histological examination, a mean reduction in epidermal thickness of 44.0% ($P < .01$) was found after acitretin treatment. This reduction was completely caused by a decrease in stratum corneum thickness; no significant reduction could be seen in the other strata (Figure 1).

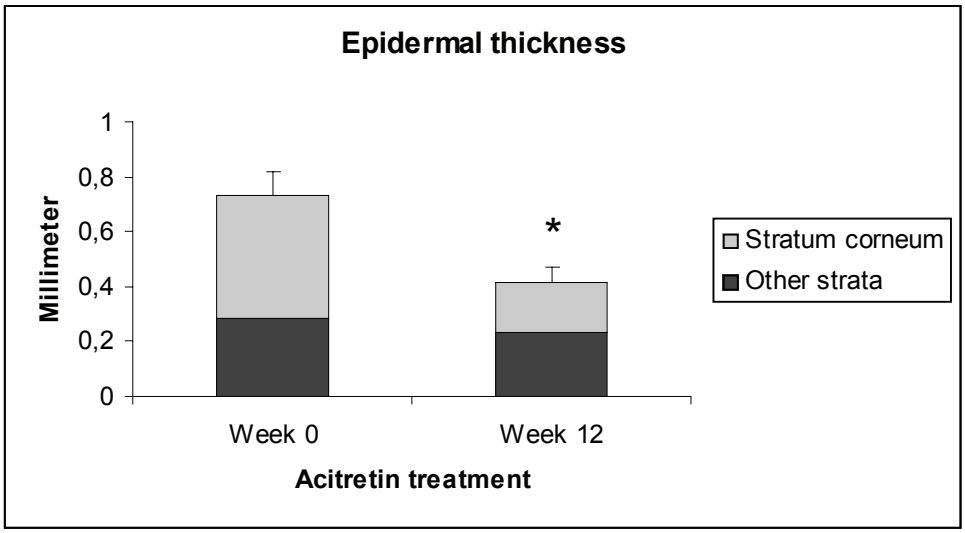

FIGURE 1:
Epidermal thickness as the sum score of stratum corneum thickness and thickness of the other strata in millimeter at baseline and after 12 weeks treatment with acitretin (mean ± SEM) (* $P < .01$; N=27).

At baseline, minimal to moderate TDI scores were found in 85% of the AKs, whereas the remaining 15% demonstrated a moderate-pronounced TDI. The infiltrates were localized perivascular and in the upper dermis. After 12 weeks of treatment, no significant alterations were seen in TDI scores (Figure 2).

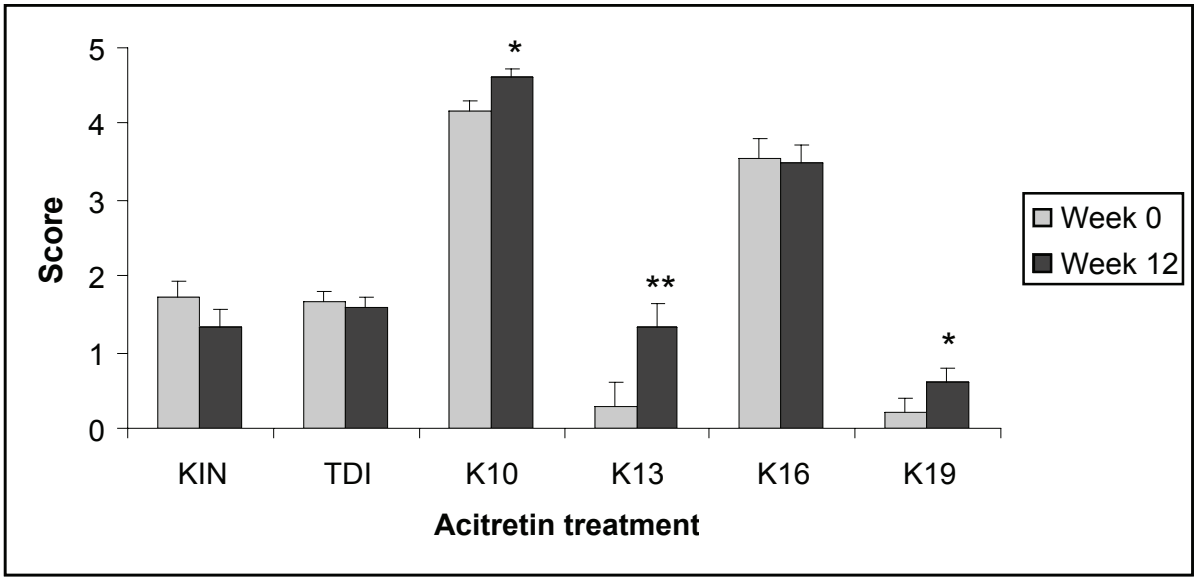

FIGURE 2:
Semi-quantitative scores for keratinocytic intraepidermal neoplasia (KIN), total dermal infiltrate (TDI), and keratins K10, K13, K16, and K19 at baseline and after 12 weeks treatment with acitretin (mean ± SEM) (* $P <$ .01; ** $P \leq .05$; N=27)

The KIN score, a scoring system for dysplasia, did not change significantly during the study period. Before treatment the KIN score was 1.7 ± 0.2 versus 1.3 ± 0.2 after treatment (Figure 2).

Immunohistochemical results

At baseline, in most AKs the epidermal proliferation parameter Ki-67 revealed numerous proliferating cells mostly in the basal cell layer, but also to a variable extent depending on KIN score in the suprabasal regions of the epidermis. Ki-67 expression was restricted to the nuclei and featured a rather homogenous staining pattern in the horizontal plane of the epidermis. Treatment with acitretin did not lead to a significant alteration in the number of Ki-67-positive keratinocytes (Figure 3). In 13 patients, an increase in Ki-67-positive cells could be demonstrated, whereas in 14 patients a decrease in this number was found after acitretin treatment.

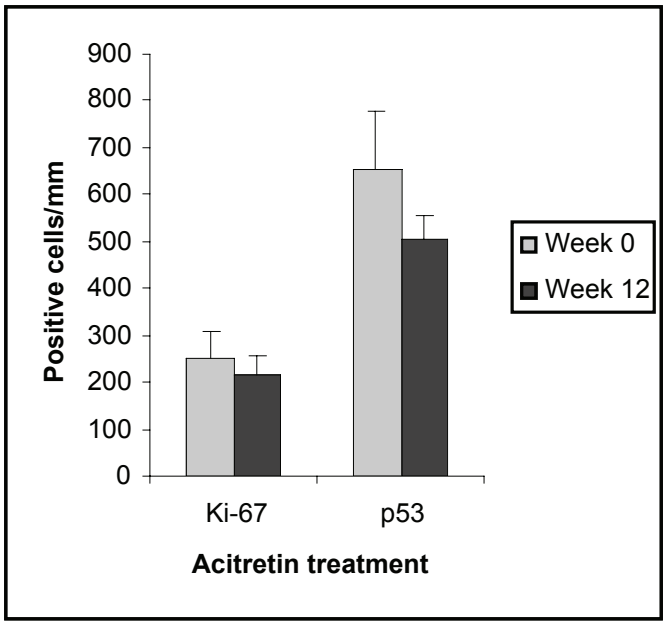

FIGURE 3:
Ki-67 and p53 scores as the number of positive stained cells per mm length of section of the slides at baseline and after 12 weeks treatment with acitretin (mean ± SEM) (N=27).

Apoptosis-associated p53 protein at baseline was mostly observed in the basal and lower suprabasal cell layers of the epidermis, but in some AKs p53 expression throughout all epidermal cell layers was seen. Expression of P53 basically followed the pattern of Ki-67. However, incomplete staining areas for p53 were often observed, whereas the corresponding Ki-67 staining in a consecutive slide was continuous along the complete length of the slide. Overall, the number of p53 positive cells was also much higher than the number of Ki-67-positive cells. Statistical analysis revealed no significant change in p53 staining after acitretin treatment (Figure 3).
Changes in the different parameters of keratinization are depicted in figure 2. Epidermal differentiation parameters at baseline showed the following patterns. Normal differentiation-associated K10 expression was only seen in the suprabasal compartment. Its staining pattern was less when compared to normal skin and often showed unstained foci. After acitretin treatment, a significant increase in K10 expression was found with a more complete staining pattern (P= .02).
Hyperproliferation-associated K16 was expressed in the suprabasal compartment of all AKs. Expression was moderate to pronounced, with staining of all suprabasal cell layers. Most intense staining for K16 was observed suprabasally in the hypertrophic center of AKs directly below the stratum corneum. The degree of K16 expression before and after acitretin treatment was similar.
In the untreated AKs, terminal differentiation-associated transglutaminase staining was seen in the stratum granulosum and stratum spinosum. The number of positive cell layers varied among the AKs from 1 to 15. In addition, also in individual AKs differences could be seen in the number of positive cell layers. In general, in the interpapillary areas the number of positive cell layers was the highest. No significant changes in transglutaminase scores could be detected after acitretin treatment.

Keratin 13 and keratin 19
At baseline, K13 expression was found in 8 patients and this was localized in the suprabasal cell layers of the AKs. The staining pattern in these sections was always sporadic with single

cell expression mostly scattered in the epidermis. After acitretin treatment K13 expression was found in 14 patients featuring increased expression in the suprabasal regions of the epidermis. No K13 staining was observed in the basal cell compartment. This increase in K13 expression (in number and score) after acitretin treatment was significant (P< .01). In Figure 4, K13 expression is shown in a non-retinoid treated actinic keratosis and in an acitretin-treated actinic keratosis.

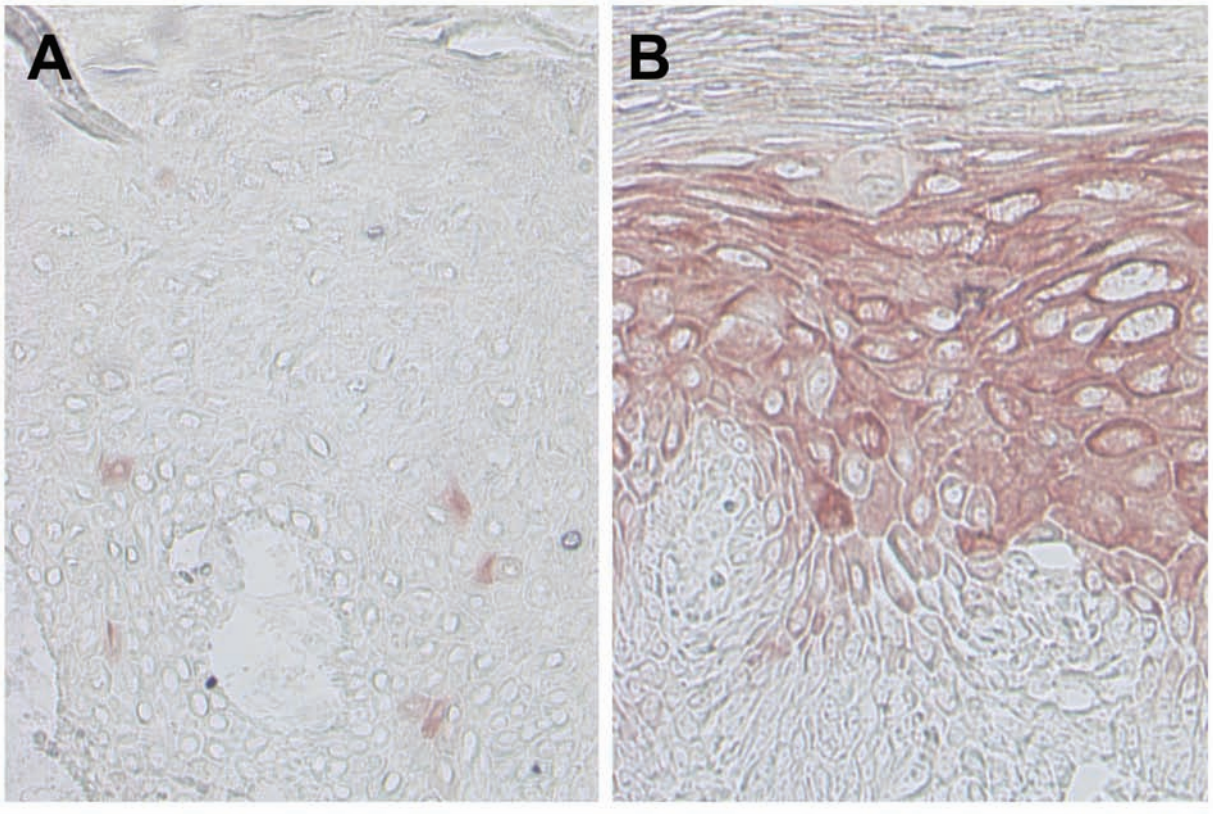

FIGURE 4:
A, K13 expression (1C7) in tissue samples of a non-retinoid treated actinic keratosis (single cell expression; X200) and (**B**) of an acitretin-treated actinic keratosis (increased expression; X200).

At baseline, in 4 patients K19 expression was found. Its grade of expression varied from sporadic to minimal, with typical single cell expression mainly located in the suprabasal compartment. After acitretin treatment K19 could be detected in 10 AKs. After treatment K19 was primarily located in the basal cell layers with minimal expression in the suprabasal compartment. The expression of K19 increased (*P*= .05) from single cell to continuous staining (Figure 5).

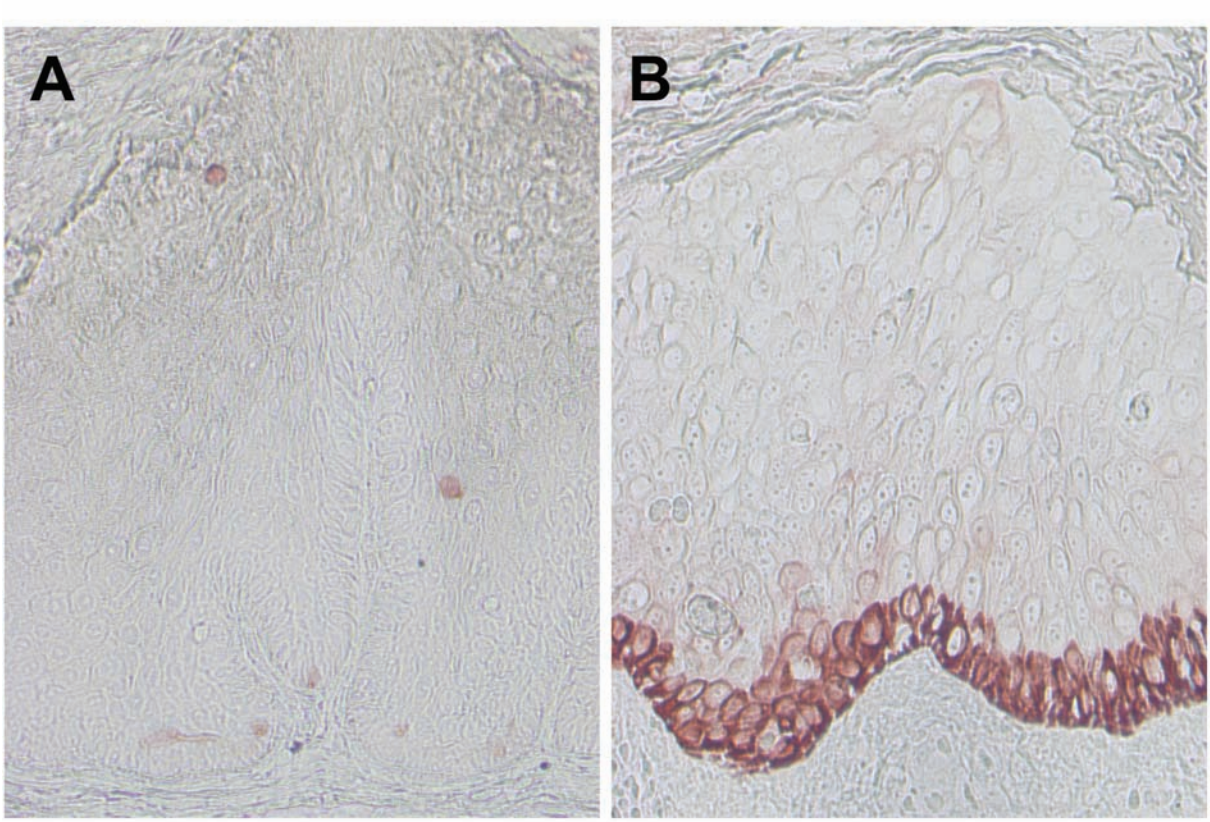

FIGURE 5:
A, K19 expression (RCK108) in tissue samples of a non-retinoid treated actinic keratosis (single cell expression; X200) and (B) in an acitretin-treated actinic keratosis increased expression (X200).

None of the patients who showed K19 expression at baseline, and only 2 out of 8 patients who showed K13 at baseline, had previously used retinoids.

Correlations

In the baseline samples, a significant positive correlation was found between Ki-67 and p53 (R=0.61; P = .001), between Ki-67 and KIN score (R=0.50; P= .013) and between KIN and p53 score (R=0.48; P= .02).

Because of the unexpected finding of K13 and K19 expression in some of the samples at baseline, we looked at correlations between K13/K19 and parameters positively correlated with malignant transformation, including epidermal proliferation (Ki-67) [146,177] , (mutant) p53 expression [146,177] , and KIN score [38], as it has been suggested that the presence of low-molecular weight proteins in human skin can be a sign of malignant transformation. In addition, we looked at a correlation between K13/K19 and the frequency of newly formed SCCs in the year before study participation.

With respect to K13 expression at baseline, significant positive correlations were found with Ki-67 (R=0.41; *P*= .036) and with the number of SCCs that each patient had developed in the 12 months immediately before treatment (R=0.43; *P*= .024).

For K19, a similar positive correlation with Ki-67 (R=0.56; *P*= .003) was found in the baseline samples as well as significant positive correlations with p53 (R=0.56; *P*= .002) and KIN score (R=0.53; *P*= .007). No statistical significant correlation between baseline K13 and K19 expression was found.

In order to assess whether induction of K13 and K19 in AKs by acitretin are correlated with each other, we compared K13 scores with K19 scores in patients who had at least grade 2 expressions in either K13 or K19 (N=10) with patients who were both K13 and K19 negative

(N=8) after acitretin treatment. Grade 1 (single cell) expression was excluded because this was also seen in untreated biopsies. After this correction for single cell expression in acitretin-treated AKs, a statistical significant correlation (R=0.49; *P*= .039) between K13 and K19 could be demonstrated in these 18 patients.

DISCUSSION

From previous studies it is known that retinoids can improve the aspect of AKs by reducing its roughness [9,73,102]. Our data indicate that this clinical improvement is caused by a decrease in thickness of the stratum corneum, whereas the other strata remain unchanged with respect to thickness or the number of cell layers. Other histological parameters (TDI, KIN) did not change significantly. With respect to immunohistochemical data, we have demonstrated alterations in K10, K13, and K19, but no effect on K16, transglutaminase, epidermal proliferation, and p53 expression. These results are in contrast to psoriasis, where a 12-week treatment period with systemic acitretin in a similar dose led to significant reduction in epidermal proliferation, transglutaminase, K16, and dermal inflammation in a group of 10 patients[112].

In general, retinoids are known to alter normal differentiation of the cornifying epidermis towards an esophageal-type of differentiation by stimulating the synthesis of low molecular weight keratins, such as K13, that are normally present in internal squamous epithelia, but not in adult human skin [57,106].

In studies with cultured keratinocytes, coupled induction of K13 and K19 by retinoids has been described [106,107]. With respect to in vivo studies, K13 expression was induced by topical application of all-*trans*-retinoic acid (ATRA) on photoaged human skin [162] and by systemic treatment in warts in RTRs [20]. To our knowledge, no in vivo studies have been performed that report an induction of K19 by retinoids. The present study in AKs in RTRs also demonstrates that induction of K13 by acitretin is correlated with K19 induction, although, in contrast to fetal skin, expression of both parameters was seen uncoupled. Uncoupled expression of K13 and K19 has been reported previously in a human SCC cell line [1] and in warts in RTRs [20]. Our data also suggest that induction of K13 in AKs by acitretin is more frequently seen than induction of K19. Earlier findings in human epidermal cultures indeed showed stronger induction of K13 than of K19 by retinoids[106].

Apart from the induction of K13 and K19 by acitretin, in the present study we found evidence that K13 expression in non-retinoid-treated AKs is correlated with higher epidermal proliferation, higher (mutant) p53 protein expression, and a higher degree of dysplasia. Therefore, these data indicate that assessment of K13 and K19 in non-retinoid treated AKs may provide further information on the risk of malignant transformation and thus may be of interest for the identification of high-risk lesions.

It is intriguing that retinoid treatment induces keratins that are correlated with malignant transformation and on the other hand cause a reduction in skin cancer incidence [9,73,102,132,160,180]. Therefore, it is likely that, with respect to K13 and K19 expression, we deal with epiphenomena, related to two distinct pathways: malignant transformation associated dedifferentiation and retinoid-associated keratinization.

KIN scores based on histological criteria did not always correlate well with the clinical picture in this group of RTRs. While all 27 baseline biopsies were taken from typical AKs with evident erythematosquamous hallmarks, suggesting a grade II to III KIN score, in 2 biopsies no dysplasia (KIN 0), and in 10 biopsies a KIN grade I was found. Therefore, in RTRs, who often have numerous erythematosquamous and hypertrophic AKs, the directly visible clinical hallmarks of these lesions may not always reflect the grade of dysplasia in the epidermis of these lesions.

Combining clinical, histological, and immunohistochemical data, it can be concluded that treatment with systemic acitretin improves AKs probably via alteration of the keratinization process of the keratinocytes, leading to peeling of the hypertrophic stratum corneum of these lesions and clinically resulting in softening of the skin. No significant decrease in proliferation and dysplasia was found, which might be the explanation why recurrence of AKs is normally seen after cessation of acitretin treatment. Expression of low-molecular-weight keratins K13 and/or K19 in non-retinoid treated AKs is correlated with parameters that are indicative for high-risk AKs. Therefore, K13 and K19 may provide two new diagnostic parameters for assessment of AKs at particular risk for transformation into a SCC.

3.3

Immunohistochemical effects of temporary cessation of long-term acitretin treatment in keratinocytic intraepidermal neoplasia of renal transplant recipients

Abstract

***Objectives**: to study effects of temporary cessation of systemic acitretin treatment in renal transplant recipients (RTRs).*

***Design**: prospective, non-randomized clinical and immunohistochemical study.*

***Setting**: University hospital.*

***Patients and methods**: 9 RTRs on systemic acitretin (mean duration 2.3±0.6 yrs; mean dosage 18.3±9.9mg) were asked to interrupt their treatment for three months. Patients gave written informed consent. Patients were seen at intervals of 6 weeks, starting at the moment of acitretin interruption. Each visit epidermal lesions were counted; erythema and desquamation of normal skin and AKs were recorded and induration of AKs, a visual analogue score (VAS) for patient's contentedness was performed, and clinical comparable AKs were biopsied.*

***Main outcome measurements**:*

1.establish change in numbers of skin lesions and skin (pre) cancers after acitretin interruption;

2.compare expression of keratin 13 (K13), MIB-1, p53 and $p16^{INK4A}$ in KIN lesions of RTRs, with and without acitretin;

***Results**: After 3 months, the number of warts was significantly increased (p=0.02) and AKs were more indurated (p=0.02). VAS score reduced significantly already after 6 weeks (p=0.04), and strongly after 12 weeks (p=0.004). We could not demonstrate a significant increase in number of AKs (p=0.09) or SCCs (p=0.3).*

After 6 and 12 weeks, a significant decrease of K13 expression was found (p=0.03 and p=0.02 respectively); zebroid K13 expression also decreased but this reduction was not significant (5/9 cases at the start vs. 1/9 cases after 6 and 12 weeks ; p=0.10). No alteration in expression of cell cycle associated markers was found.

***Conclusions**: Acitretin withdrawal in RTRs leads to*

1.clinical deterioration within 3 months, without significant increase in skin (pre) cancer, and

2.significant reduction of aberrant K13 expression, without alteration in expression of cell-cycle-associated markers.

INTRODUCTION

Retinoids, synthetic vitamin A analogues, interfere with epidermal growth and differentiation. These effects of retinoids on proliferation and differentiation make retinoids of interest in the treatment of hyperproliferative skin diseases, such as psoriasis, disorders of keratinisation and as an anti-tumor drug [197].

Renal transplant recipients (RTRs) in general develop multiple warts, actinic keratoses (AKs) and non-melanoma skin cancers (NMSCs), especially on sun-exposed parts of the body[24,26,117], which lead to a major co-morbidity. In the past two decades, systemic treatment with retinoids has shown to be promising in chemoprevention of actinic keratoses (AKs) and squamous cell carcinoma (SCC)[118], also in RTRs [9,73,132,180,203].

Several studies suggested a beneficial effect of systemic retinoid treatment, with either etretinate or acitretin, with prevention of SSCs and reduction of the incidence of keratotic lesions in RTRs with NMSC in their history or severe lesions [9,73,118,180,203]

However, after stopping the retinoid treatment the beneficial effect disappeared, with report of relapses varying from 4-12 months after discontinuation of retinoid therapy [9,46,118,203]. Therefore, some have recommended continuous treatment [132].

How retinoids exert their chemopreventive effect is so far unknown. Recently, we demonstrated that retinoids alter differentiation in almost 90% of warts of RTRs, with induction of keratin 13 (K13) expression [20], a keratin normally not present in the adult epidermis [28]. A specific zebroid K13 expression pattern, proved indicative of retinoid bioactivity in skin of these patients. Whether comparable K13 expression patterns are present in retinoid treated KIN lesions of RTRs remains to be established.

In addition, in view of the clinical efficacy of retinoids in NMSCs in RTRs, it is feasible that retinoids interfere with cell cycle associated proteins. Thus far, effects of retinoids on expression of cell cycle associated markers in KIN lesions of RTRs have not been published. The most current and so far best documented cell cycle associated marker for intraepithelial neoplasia (especially in neoplasia of the uterine cervix) is the proliferation marker, MIB-1 [70,127] .In AKs of both immunocompetent individuals and RTRs frequent overexpression of the p53 tumor suppressor is reported [66,120]. Recent data show that besides the p53 pathway, also the pathway involving the tumor suppressor $p16^{INK4A}$ is involved in skin carcinogenesis [188]; in high grade KIN lesions of both RTRs and immunocompetent individuals we found frequent $p16^{INK4A}$ overexpression (own data, manuscript in preparation).

The present prospective study was designed to study the effects of 3 months withdrawal of long term acitretin treatment on clinical skin condition and skin (pre) cancer development in RTRs, and assess effects on histology and immunohistochemical expression of the three biomarkers MIB-1, p53 and $p16^{INK4A}$ in KIN lesions of RTRs. Furthermore, we examined whether K13 expression in acitretin treated KIN lesions showed comparable expression pattern as previously found in warts during retinoid treatment, and if K13 expression reduced after acitretin withdrawal.

MATERIALS AND METHODS

Patients

RTRs on systemic acitretin (mean duration of acitretin treatment 2.3+/-0.6yr; mean dosage 18.3+/-9.9 mg/day) were asked to interrupt their treatment for 3 months.

Initially, 11 out of 16 approached patients agreed to participate and in all cases written informed consent was obtained. However, one female withdrew after the first visit due to interfering breast cancer and 1 patient withdrew because of progression of AKs and SCCs, 6 weeks after the start of the study.

The characteristics of the remaining 9 patients (3 males, 6 females) that finally completed the 3-month study period, including data on previous retinoid therapy and type and duration of immunosuppression are listed in Table 1.

Table 1. Characteristics of the 9 participating patients; M=male; F=female; yrs=years; A=azathioprine; P=prednisolone; S=sirolimus; AK=actinic keratoses.

patient	Age (yrs)	sex	Duration imm.suppress. (yrs)	Type imm.suppression	Dosage acitretin at T0(mg/day)	Dosage acitretin at T0 (mg/kg)	Duration of acitretin treatment(yrs)
1	65	M	33	AP	20	0.24	2.9
2	60	F	6	AP	35	0.30	2.9
3	53	F	27	P+S	20	0.29	2.8
4	64	M	8	AP	10	0.20	1.5
5	64	F	24	AP	15	0.16	2.6
6	48	F	22	AP	15	0.15	2.6
7	60	M	21	AP	15	0.19	2.3
8	51	F	26	C	25	0.26	1.9
9	60	F	13	AP	10	0.13	1.5
Mean (±SD)	58.3		20 (±9.1)		18.3 (±9.9)	0.21(±0.06)	2.3 (±0.6)

Design of the study

During the 3-month study period the patients were seen at three defined moments, with intervals of 6 weeks in between each visit. At time T0 the study started and on this day systemic acitretin was stopped. Control visits were respectively 6 (T1), and 12 (T2) weeks after acitretin stop.

At each visit all warts and actinic keratoses in each patient were counted and scored by the same dermatological investigator, familiar with this type of patients and their skin lesions. Erythema and desquamation of lesional and non-lesional skin and induration of lesional skin were judged, using a scale of 0-3 (0=absent, 1= minimal, 2=moderate, 3=severe).

Patients were asked to give a subjective judgment of their skin condition using a visual analogue score (VAS), with a scale from 0-10 (0=very discontented, 10=very satisfied). At the end of each visit a 6 mm skin biopsy was taken; it was attempted to biopsy clinically identical (at baseline defined) actinic keratoses from the same location in all three sessions, preferentially from the forearm.

Of each participating RTR, the number of biopsied AKs, Bowen's diseases (BDs) and SCCs during the 3-month study period was compared with the number of lesions, which had occurred in the last 3 months with retinoid treatment.

Histopathology and immunohistochemistry

A KIN (keratinocytic intraepidermal neoplasia)-classification was assigned to all skin biopsies [38,216].

Immunohistochemical analysis was performed on all lesions using standard avidin-biotin-peroxidase complex system with diaminobenzidine (DAB) as the chromogen. In brief, 4-micron thick paraffin sections were deparaffinized, hydrated, and washed in buffered saline phosphate.

The monoclonal antibodies used, the antigen they recognise, and pretreatment and dilutions are listed in Table 2.

Table 2. Antibodies with the recognized antigens, used dilution, pretreatment, incubation time and temperature.

antibody	antigen	Source	dilution	pretreatment	Incubation time and temperature
DO7	P53	Neomarkers	1:400	microwave	4^0C overnight
$P16^{INK4A}$/MTS1, Ab-4, 16PO4	$P16^{INK4A}$	Neomarkers	1:100	microwave	4^0C overnight
MIB-1	Ki-67	Progen	1:100	microwave	4^0C overnight
2D7 (IgG2b)	Keratin 13	G. van Muyen	1:1	microwave	4^0C overnight

After incubation with primary antibodies, sections were incubated for 30 minutes with (a 1: 200 dilution of) biotinylated horse anti-mouse (Vector laboratories, Burlingham, CA), followed by 45 min. incubation with (a 1:50 dilution of) avidin-biotin complex (Vector laboratories, Burlingham, CA).
Sections were counterstained with Mayer's haematoxylin.
Immunoreactivity was scored semi-quantitatively for all applied immunohistochemical markers, in the following manner:
MIB-1 and p53 : for both staining was nuclear. Staining was scored as previously published by Keating et al. in cervical neoplasia [99]: 0= only basal layer positivity, 1=positivity confined to basal 1/3 of epidermis, 2=positivity confined to basal 2/3 of epidermis or 3= transepidermal positive staining).
K13 : staining was cytoplasmic. Scoring was performed as described previously [20]; 0=negative staining, 1= suprabasal single cell positivity, 2= zebroid pattern.
$P16^{INK4}$:staining was both nuclear and cytoplasmic. Staining was scored as for MIB-1 and p53.
Scoring of immunostaining was performed without knowledge of patient history.

Statistics

The Lilliefors test for normality disclosed that the data were not-Gaussian distributed. Therefore, non-parametric statistical procedures were used to analyze the data. Since the small series of patients included in this study the exact significance level is given or an unbiased estimate of exact significance level, calculated by repeatedly sampling (= Monte Carlo method).
The relations between the grade of KIN and expression of K13, MIB1, p53 and $p16^{INK4A}$ were assessed with both rank correlation analysis and the Jonckheere-Terpstra test, a distribution-free test for ordered alternatives (Hollander and Wolfe).
In this study a before-after-design [176] was used to study the effect of cessation of retinoid treatment on some clinical and immunohistochemical parameters. If a significant effect was present after 6 weeks (T1) it will be further mentioned a short-term effect. If a significant effect was present after 13 weeks (T2) it will be further mentioned a long-term effect.
A partial rank correlation analysis controlled for KIN grade was used to assess the effect of retinoid treatment cessation on the KIN-grade dependent immunohistochemical features.
For the KIN-grade independent immunohistochemical features and clinical parameters the following statistical procedures were used to analyze the treatment cessation effect.
Two planned pair wise comparisons (T1 versus T0 and T2 versus T0) were used to study the short and long term effects. The Wilcoxon matched-pairs signed-rank test and McNemar test were used for these pair wise comparisons. The latter test was used for the dichotomous dependent variables. With a limited number of planned pair wise comparisons the Bonferroni

adjustment of the Type I error rate can be omitted [176]. Because there is a definite expectation about the direction of the therapy cessation effect, a directional (one-tailed) alternative hypothesis was used.
All statistical analyses were performed with SPSS 10.0 for Windows.

RESULTS

Clinical effects of interruption of systemic acitretin

The number of counted warts and AKs at each control visit is listed in Table 3. Only for the number of warts a significant increase was seen after 3 months when compared to the start of the study (p=0.02); for AKs no significant differences in number of lesions were found.
Desquamation of clinically normal and lesional skin did not significantly alter during the 3-month study period (Table 3). Erythema of normal skin reduced significantly already after 6 weeks (p=0.03): induration of AKs increased significantly only in the first 6 weeks after acitretin withdrawal (p=0.004): after 3 months this increased induration was no longer significant when compared to the start of the study.
The mean VAS-score of all patients at each visit is listed in table 3; already after 6 weeks of acitretin VAS score is significantly reduced (p=0.04), and after 12 weeks this effect is strongly significant (p=0.004).
During the 3-month study period, patients developed 7 new AKs, 2 BDs, 5 SSCs and 1 basal cell carcinoma (BCC), compared to 2 new AKs and 2 SCCs in the 3 months pre-study period while still on acitretin therapy; differences were not significant, neither for the number of AKs (p=0.09), nor for the number of SCCs (p=0.3). However, numbers are small and follow-up is relatively short.

Table 3. T0=start of the study; T1=6 weeks after acitretin withdrawal; T2=12 weeks after acitretin withdrawal;p1=p value T0-T1(week0-6); p2=p value T0-T2(week 0-12); SEM=standard error of mean; AKs= actinic keratoses; VAS=visual analogue score. Figures in bold indicating statistically significant findings.

parameter	T0 mean±SEM	T1 mean±SEM	T2 mean±SEM	P1	P2
number of warts	1.6±0.8	1.9±1.0	2.7±1.1	0.5	0.02
number of AKs	129.6±36.4	108.1±26.7	109.6±25.4	0.1	0.2
Erythema of perilesional normal skin	0.9±0.3	0.2±0.2	0.2±0.2	0.03	0.03
Desquamation of perilesional normal skin	0.6±0.2	0.3±0.2	0.3±0.2	0.3	0.3
Erythema AK	1.9±0.4	1.7±0.3	1.7±0.3	0.4	0.4
Desquamation AK	1.1±0.3	2.00±0.3	1.6±0.4	0.05	0.3
Induration AK	0.9±0.2	2.0±0.2	1.9±0.3	0.004	0.05
VAS	5.6±0.7	4.4±0.7	3.1±0.6	0.04	0.004

Histopathologic and immunohistochemical effects of interruption of systemic acitretin

Since immunohistochemical parameters can be dependent of KIN grade, we had to take this into account while studying the effects of cessation of acitretin. In total 27 biopsies, 3 of each participating RTR were processed for histology and immunohistochemistry. Of each patient three biopsied were taken at T0, T1, and T2 that came from clinically comparable lesions, which on histological examination sometimes varied in KIN grade. In 8 patients in all visits either KIN 1 or 2 was biopsied, in 1 patient each control visit a KIN 3 lesion was biopsied. A correlation analysis showed that MIB-1 score strongly correlated with KIN grade (r=1). All

KIN 1 lesions showed a 1+ MIB-1 score, all KIN 2 lesions a 2+ and all KIN3 lesions a 3+ MIB-1 score. The p53 expression was weakly correlated with KIN grade (r=0.5, p=0.01). Expression of both p16 and K13 were not significantly correlated with KIN grade (r=0.33, p=0.10 and r=-0.03, p=0.90 respectively).
When taken the KIN grade into account, we found no significant correlation between the biopsy number and the expression of MIB-1, p53 and $p16^{INK4A}$. Therefore, in this small patient group no influence of acitretin withdrawal on expression of these three biomarkers could be demonstrated. However, a negative correlation between K13 expression and the time of biopsy (r=-0.50, p=0.01) was present.
K13 expression decreased significantly from 1.44±0.24 to 0.33±0.24 after 12 weeks (p=0.02), when comparing K13 negative versus K13 positive cases. At the start of the study 90% of KIN lesions were K13 positive, after 6 weeks 33% and after 12 weeks 22% (Figure 1A). If we only considered *zebroid* expression, there was clearly a trend towards decrease of K13 expression after acitretin withdrawal (p=0.10): at the start of the study zebroid K13 expression was found in 5/9 patients (56%), while after 6 weeks and 12 weeks of acitretin withdrawal zebroid K13 expression was only present in 1/9 patients (11%, Figure 1 B). The patient at 6 and 12 weeks with zebroid K13 expression was not the same patient.

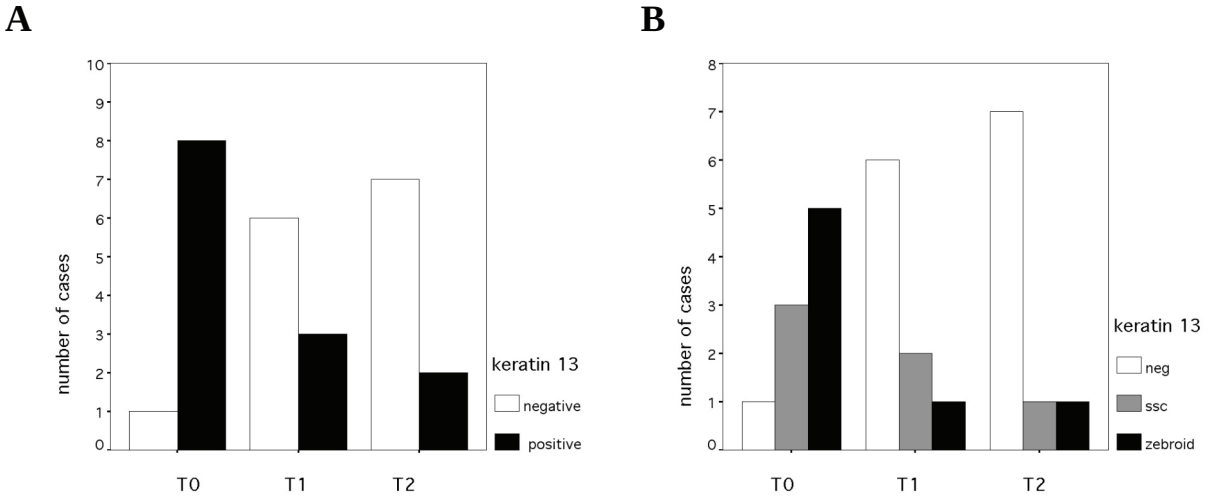

FIGURE 1:
Expression of keratin 13 (K13) in keratinocytic intraepidermal neoplasia lesions of renal transplant recipients at baseline(T0), 6 weeks (T1), and 12 weeks (T2) after withdrawal. of acitretin therapy.
A, Keratin 13-positive vs. K13-negative cases. Keratin 13 expression decreased significantly 12 weeks after withdrawal of acitretin therapy (*P* =0.02).
B, Keratin 13-positive cases subdivided into cases having suprabasal single cell (SSC) expression or zebroid K13 expression. The latter pattern was previously found to be correlated with retinoid treatment [20]. For zebroid K13 expression, a decreasing trend was present, with zebroid K13 expression being present in 5 (56%) of 9 patients at T0, and only in 1 patient (11%) after 6 weeks and 12 weeks (*P* = 0.10).

Histopathology of the skin biopsies at the start of the study with a zebroid K13 pattern, revealed in the areas of immunohistochemical K13 expression often compact hyperorthokeratosis, hypergranulosis, acanthosis and swelling with focally cytoplasmic vacuolization of the keratinocytes when compared to the K13 negative adjacent epithelium (fig. 2). These are known histological changes following topical and systemic retinoid treatment [56]. The transition with the K13 negative epithelium was mostly sharp and abrupt.

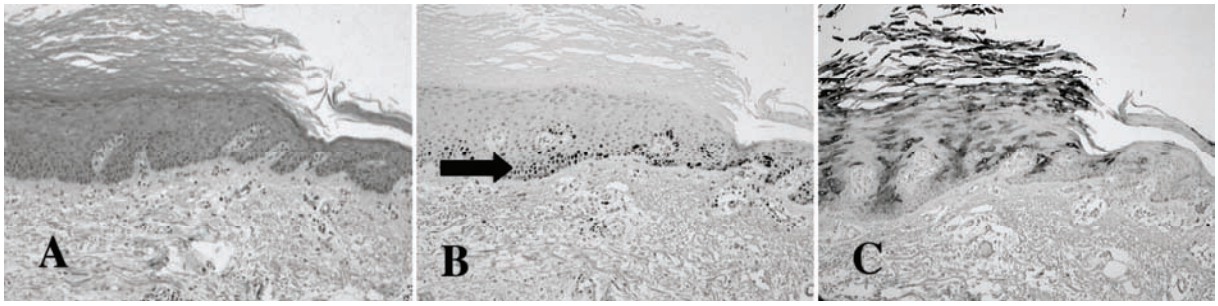

FIGURE 2:
A, Keratinocytic intraepidermal neoplasia (KIN) lesion of a renal transplant recipient, with biopsy specimen obtained at the start of the study (first day of acitretin withdrawal). Findings are consistent with KIN 1. An abrupt transition from normal to lesional skin with hyperorthokeratosis and acanthosis is seen; a slightly disordered epidermal structure with atypia in the basal one third of the epithelium is present (hematoxylin-eosin, original magnification / 100).
B,.MIB-1 shows positive nuclear staining in the basal one-third layer of the epidermis (arrow). Findings are consistent with KIN 1 (MIB-1, immunohistochemical analysis by using standard avidin-biotin-peroxidase complex system with diaminobenzidine, original magnification / 100).
C, In lesional skin, a zebroid keratin 13 (K13) expression pattern is present with alternating columns of K13-positive and K13-negative cells (K13, immunohistochemical analysis by using standard avidin-biotin-peroxidase complex system with diaminobenzidine, original magnification / 100).

DISCUSSION

The present study shows that 3 months cessation of systemic acitretin treatment is followed by reduction of aberrant differentiation in KIN lesions of RTRs with significant decrease of K13 expression. Expression of the proliferation marker MIB-1, and of cell-cycle-associated proteins p53 and p16, all often found overexpressed in KIN[120,146] (p16, own data), were not altered on acitretin withdrawal. These data suggest that retinoids in RTRs exert their effect by inducing aberrant terminal differentiation (induction of K13, normally present suprabasal in internal stratified squamous epithelia, like the esophagus [185]) and not by decreasing proliferation or influencing tumor suppressors in cutaneous carcinogenesis. Previously, we found a particular *zebroid* K13 expression pattern related to retinoid treatment in *warts* of RTRs which we postulated to be indicative of retinoid bioactivity in warts of these RTRs [20]; almost 90% of *warts* in *RTRs* showed this typical zebroid K13 pattern in case of retinoid treatment.
In the present study, concerned with *KIN* lesions, we found a much lower percentage of zebroid K13 pattern at the start of the study, than would be expected from the previous findings in warts; only 5/9 patients (56%) on acitretin showed zebroid K13 expression. This zebroid K13 expression markedly reduced on acitretin withdrawal, with only 1/9 patients (11%) displaying this zebroid K13 pattern after 6 and 12 weeks, as we already anticipated based on the previously found correlation between zebroid K13 expression and retinoid treatment in warts. This marked reduction, however, proved not significant ($p=0.1$), probably due to the small number of patients. When combining the two patterns of K13 expression, the decrease in K13 expression reached significance after 6 and 12 weeks of acitretin withdrawal ($p=0.03$ and $p=0.02$ respectively).
The lower frequency of zebroid K13 expression presently found in *KIN lesions* when compared to *warts*, might be attributable to the dysplastic changes in KIN with genetic alterations that might interfere with the retinoid receptor or interactions with the retinoid receptor or alterations in keratin gene regulation (e.g. K13) that would otherwise have been

transcriptionally induced. Indeed, recently Xu et al. found progressive decrease in nuclear retinoid receptors during skin squamous carcinogenesis [215]. On the other hand in warts, (transforming) types of human papillomavirus, might increase the sensitivity of keratinocytes to retinoid effects [152]. Future research on warts and AKs within one patient during acitretin treatment might elucidate whether there is an interlesional variation in retinoid susceptibility.

In parallel to the immunohistochemical decrease in K13 expression, clinical progression of skin lesions was seen with significant increase in warts ($p=0.02$). We could not demonstrate a significant increase in number of AKs and SCCs when acitretin treatment was interrupted for 3 months. This could be caused by the relatively small number of patients and the short study period. The latter possibility is supported by the previous studies by Bouwes Bavinck[27], where a relapse of new skin cancers occurred between 3 and 6 months after acitretin withdrawal, and by Shuttleworth[180], who described the development of further lesions 6 months after etretinate cessation.

VAS score also significantly decreased in the 3 months study period, reflecting a reduction in contentment of the patients with their skin condition.

Already after 6 weeks of acitretin withdrawal, erythema of normal perilesional skin diminished significantly, as could be expected since erythema is a well-known adverse effect of topical and oral retinoid treatment [118]. After 3 months a trend towards increased induration of AKs was present; since we found no significant concomitant increase in SCCs, induration seems more likely due to increased hyperkeratosis (in the absence of retinoids) instead of infiltrative growth. Retinoids could have an antihyperkeratotic, by induction of aberrant suprabasal K13 expression causing terminal differentiation towards internal *non-keratinizing* stratified squamous epithelia [185]. This diminished hyperkeratosis with retinoids leads to better cosmetic appearance of the skin, important for patient's contentment. Increased induration in the absence of retinoids could make it more difficult for the clinician to differentiate AKs from invasive lesions (masking effect), necessitating more frequent biopsies in these RTRs, which are already subject to frequent skin biopsies.

So far, effects of systemic retinoids on epidermal proliferation in RTRs were only studied by means of ^{3}H thymidine labeling index; Shuttleworth et al. [180] found a significant increase in the labeling indices after 6 months of systemic etretinate treatment in normal sun-exposed skin, but not in sun protected skin in 6 RTRs; effects on dysplastic skin were not assessed. Besides Gibson et al., who found no significant differences in histological features of SCCs [73], no other studies on chemoprevention by systemic retinoids in RTRs [27,160,203] included histological or immunohistochemical evaluation of retinoid effects on skin lesions, to study for instance effects on proliferation or differentiation.

In the present study, clinical comparable AKs were biopsied, which on histological examination sometimes varied in KIN grade. When taken the KIN grade into account, we found no significant correlation between the time of biopsy and the expression of MIB-1, p53 and $p16^{INK4A}$. Therefore in this small patient group no influence of acitretin withdrawal on expression of these three biomarkers could be demonstrated.

In conclusion, this study demonstrates that 3 months cessation of systemic acitretin therapy in RTRs, leads to significant reduction of aberrant K13 expression, without alteration in expression of proliferation and cell-cycle associated markers (MIB-1, p53, $p16^{INK4A}$), in KIN lesions of RTRs. Therefore, our data imply that if retinoids in RTRs exert an anti-neoplastic effect, it is by modifying differentiation (K13 expression).

Chapter 4

SUMMARY, SUPPLEMENTARY DISCUSSION AND FUTURE PERSPECTIVES

The central theme of this thesis is the role of cell- cycle associated proteins, especially tumor suppressor proteins p53, p16, and p14, in the pathogenesis of skin cancer focused on renal transplant recipients. The objective was to gain a deeper understanding of the role of etiological factors in skin cancer development in these patients.
The second aim was to study feasibility of p53 and INK4a-ARF mutation analysis in determination of primary skin cancer in case of metastasis.
The third objective was to investigate the mode of action of retinoids in skin cancer prevention in renal transplant recipients.
The first 2 objectives were dealt with in chapter 2, the third in chapter 3.
In this chapter the results of the performed studies will be briefly summarized and discussed.

4.1

Tumor suppressors p53, p16 and p14 in cutaneous carcinogenesis; the influence of sun-exposure, transplantation and human papillomavirus (HPV)1

Sun exposition is a major etiological factor in the development of cutaneous squamous cell carcinoma (CSCC) and its precursors, keratinocytic intraepidermal neoplasia (KIN). Previous studies have demonstrated UV-related mutations in the tumor suppressor gene TP53 in these epidermal (pre) malignant lesions. More recently also UV-related mutations in the CDKN2A (INK4A-ARF) locus, which encodes for two tumor suppressor proteins p16 and p14, were found in epidermal neoplasia.

In immunocompetent individuals (ICIs), KIN lesions and CSCCs frequently show overexpression of the tumor suppressor proteins p53 and p16. Expression of p14 in skin (pre) malignancies is so far hardly studied.

Renal transplant recipients (RTRs) are known for developing large numbers of skin tumors, especially SCCs, which start to develop several years post-transplantation and with time their number tends to increase exponentially. These SCCs are often preceded and accompanied by large numbers of cutaneous warts and hyperkeratoses. The latter histological often represent KIN lesions and therefore are preneoplastic. All these skin lesions develop on sun-exposed sites, are more frequent in patients in warmer climates, and are more frequent in patients with a past history of high sun-exposure. These data suggest that also in RTRs sun-exposure forms a major causal factor in cutaneous carcinogenesis. Previous studies have reported UV-related p53 mutations in CSCCs of RTRs (most often low patient numbers tested with a wide range in prevalence of p53 mutations varying from 8-43%). Also p53 overexpression in (pre) malignant skin tumors in these patients has been reported, sometimes with comparable findings as in the general population. Others reported more frequent p53 overexpression in skin lesions of RTRs.

Data on p16 and p14 mutations and expression of these two proteins in skin (pre) malignancies in RTRs are currently lacking. At the moment there is only one study performed in immunosuppressed patients that were not further specified.

Because RTRs develop skin cancer at an increased rate and with a much higher frequency when compared to the general population, and because the CSCCs in these RTRs tend to behave more aggressively, there seem to be additional factors besides UV in this patient group that attribute to this enhanced cutaneous carcinogenesis. One can think of the immune suppressive treatment in these RTRs, which creates a state of decreased immune surveillance and insufficient eradication of precancerous lesions. In addition immunosuppression could increase the susceptibility for infection with oncogenic viruses. In skin tumors the mostly likely candidate oncogenic virus would be the Human Papiloma Virus (HPV), since skin tumors in RTRs are often accompanied by (HPV-induced) warts and histological KIN lesions and CSCCs in these patients are reported to contain viral features known to be caused by HPV such as koilocytosis and verrucous architecture.

In carcinoma of the uterine cervix and precursors, the role of high-risk mucosal types HPV has already been established. In these tumors integration of high risk HPV-DNA in the host genome leads to enhanced expression of the E6 and E7 oncoproteins. The E6 oncoprotein leads to inactivation of p53 and E7 causes functional activation of pRb, thereby disrupting the two major pathways important for cell cycle control, the p14-p53-MDM2- and the p16-CDK4/6-Rb pathway, causing malignant progression and enhanced cell proliferation.

At present the role of HPV in cutaneous carcinogenesis remains to be established. In contrast to cervical (pre) cancer, in skin cancer integration of HPV is rare. Additionally, in vitro studies have shown that the role of the oncoprotein E6 of presumed oncogenic HPV types in cutaneous carcinogenesis seems different from that in cervical cancer being unable to promote p53 degradation but instead might promote proteolysis of another pro-apoptotic protein, named bak.

In **chapter 2** the research aims were to assess the influence of risk factors for cutaneous carcinogenesis (sun exposure, HPV, and immune status) in RTRs compared to ICIs, and to explore whether molecular tools aid in identifying the correct primary cutaneous squamous cell carcinoma in case of metastases in patients with multiple primary skin cancers.

For this purpose, immunoexpression of the tumor suppressor proteins p53, p16, and p14, in KIN lesions and CSCCs of RTRs were compared with expression profiles in ICIs. . By comparing profiles with data in the literature on profiles in HPV induced cervical neoplasia the role of HPV in skin cancer development of these patients might become clearer. In addition PCR for mucosal HPV types was performed and related to the expression of these markers.

Finally, p53 and INK4a-ARF mutation analysis of primary tumors and their metastases were performed in order to determine usefulness of this mutation analysis in identifying primary CSCCs in case of metastases and multiple primary CSCCs.

In **2.1** it was shown that overexpression of both p53 and p16 is frequently present in skin (pre) malignancies: in 74/86 lesions (86%) p53 was expressed and in 63/86(76%) lesions p16 expression was present. Negativity for both p16 and p53 was found in 4/86 (5%) cases, while combined p53/p16 staining was most prevalent (55/86 lesions, 64%). P16 staining proved independent of p53 expression (p=0.8), and immune status, sun exposure and histological diagnosis (LKIN-HKIN-SCC) had no influence on this independency.

The study in **2.2** showed P14 expression in a total of 42/105 KIN lesions and CSCCs (40%). P14 staining was generally more difficult to detect in slides when compared to p16 or p53 staining due to often low number of positive lesional cells and nucleolar staining pattern.

P14 expression proved independent of the expression of both p53 and p16, and immune status, sun-exposure and histological diagnosis had no influence on this independency.

Together, these observations suggest that p16, p53, and p14 are all involved in epidermal carcinogenesis but that expression levels of these three proteins are independent of etiologic factors (sun exposure and immune status). Therefore, RTRs seem to use comparable pathways in skin cancer development as ICIs despite differences in immune status, but their susceptibility and rate to develop SCCs might well be determined by the deficient immune system.

Transplantation was associated with p53 expression in SCCs (p=0.02; power = 34%) caused by higher prevalence of p53 negative SCCs in RTRs than in ICIs (30 versus 0%). This suggests that in advanced stages of epidermal neoplasia in RTRs, p53 is differently involved when compared to ICIs, which might be attributable to HPV (since in cervical cancer high-risk HPV causes p53 inactivation).

Furthermore, the study in **2.1** showed that in high-grade KIN lesions (HKINs), p16 was more frequently positive than in low-grade KIN lesions (LKINs) (p=0.003; power = 49%) and SCCs (p=0.03; power = 53%). HKINs showed more frequent transepidermal p16 and p53 staining than LKIN lesions ($p < 0.001$;power >= 99%). These results with respect to strong and transepithelial p16 overexpression in HKIN are comparable to previous findings reported in literature, especially with respect to squamous cell carcinoma in situ.

The strong p16 overexpression in HKIN lesions could be caused by upregulation of wild type p16 or it could be due to p16 mutation, in analogue to p53 overexpression, which is often caused by p53 mutation. Previous limited research on p16 immune-expression and correlates

with underlying molecular changes in skin (pre) malignancies have not shown a clear cut correlation between p16 expression pattern and underlying molecular changes. In one study in CSCCs in 64% of cases there was concordance between the presence/absence of p16 protein expression and genetic results (mutation, methylation). In 70% of tumors with biallelic events, p16 protein expression was absent. In chapter **2.3** besides mutation analysis we performed immunohistochemistry for p53, p16 and p14 on the metastasized CSCCs. In this limited number of cases 5 out of 8 cases contained a p53 mutation, of which 2 showed strong (>50% + tumor cells) p53 immune expression, and 2 were p53 negative. P16 mutation was present in 5/8 cases, of which 2 tumors showed more than 10% positive lesional cells with 3 cases having absent p16 expression. Of the 3 p14-mutated carcinomas, one was strong p14 positive and 2 were p14 negative. These data were not further shown in the article.
Future studies regarding the exact role of these tumor suppressors in skin malignancies should combine analysis of protein expression patterns with molecular data. It would be interesting to study the role of p16 in the progression of HKIN into CSCCs since in carcinomas decrease of p16 expression is noted compared to HKINs.
In the archival study described in chapter **2.3** metastatic CSCCs were found to have p16 mutations in 63% (5/8 cases) of all cases. P14 mutations were present in 38% of all cases (3/8 cases). Although the patient number is limited, the frequency of p16 mutations in metastases we found is higher than that reported for primary CSCCs in both sporadic and xeroderma pigmentosum-associated SCCs (15-33%). Larger studies are needed to elucidate a potential role for p16 mutations in predicting metastatic potential of CSCCs. Maybe p16 mutation is predictive of a more aggressive behavior of the tumor with higher risk for metastasis.
In chapter 2.3 we found that mutation analysis of p53 and INK4a-ARF was useful in identifying the responsible primary tumor in case of metastasis and multiple primary CSCCs. In 7 of /8 metastases (88%) either an INK4a-ARF (6 of 8 cases) and /or p53 (3 of 8 cases) mutation was present. In 6 of 7 cases the corresponding primary could be identified by an identical mutation in p53 and/or INK4a-ARF. In the future this type of mutation analysis in patients with multiple CSCCs could attribute to correct identification of primaries responsible for metastasis, after which comparison (histological, clinical, molecular and array techniques) of these tumors with matched non-metastasizing cases might yield risk factors or a risk profile for metastatic behavior. This study furthermore showed the relevance of optimal histological processing and if possible freezing tumor tissue for research in a department of pathology. In 6 out of 14 cases of metastasized CSCC, the DNA quality of paraffin embedded tissue was insufficient to perform PCR reactions.
Finally, in chapter 2.2 detection of mucosal HPV was performed in KIN lesions and CSCCs using a short PCR fragment (SPF-LiPA) assay, which allows simultaneous detection and typing of 25 mucosal HPV genotypes. Only 2/105 specimens, both HKIN lesions in ICIs, contained HPV X and none of the 25 known mucosal genotypes was detected. Due to this low number of HPV containing cases, no relation between presence of HPV and expression of the proteins p53, p16, and p14 could be determined. We conclude that our data do not support a role for mucosal HPV types in cutaneous carcinogenesis. Future studies including cutaneous HPV types, especially epidermodysplasia verruciformis associated HPV types which are presumed to be oncogenic, in relation to expression of the three oncoproteins (together with molecular analysis) studied in this chapter are of interest in order to further elucidate the exact mechanisms by which these HPV types could be involved in skin cancer.

Retinoid treatment of benign and (pre)malignant epidermal tumors of renal transplant recipients

Several studies have demonstrated a beneficial effect of systemic retinoid treatment with either acitretin or etrinitate with respect to chemoprevention of skin(pre)malignancies in renal transplant recipients (RTRs). Since these patients often have multiple KIN lesions and CSCCs, which affect large areas of the body surface, systemic treatment is an important treatment modality. A number of studies showed that the effect of retinoid treatment was only temporarily with even rebound and enhanced tumor development after therapy cessation.

In none of the studies concerned with systemic retinoid treatment in RTRs, histology of (peri)lesional skin or immunohistochemistry with markers for instance proliferation (MIB-1/Ki-67) or markers for apoptosis and cell-cycle associated proteins (p53/p16) were performed to elucidate the chemopreventive action of retinoids.

Since retinoids seem to decrease skin tumor development, one could hypothesize that retinoids influence differentiation and/or proliferation and/or apoptosis of keratinocytes.

Data from animal and in vitro studies with cultured human keratinocytes have indicated that retinoids repressed expression of epidermal differentiation-specific keratins (K1/K10) and strikingly reinduced expression of two lower molecular weight keratins, corresponding to K13 and K19 respectively. Since these two keratins are coexpressed in fetal skin, this type of retinoid-induced differentiation is also termed embryonic differentiation. Furthermore retinoids in vitro increase the keratins K4, K15 and the hyperproliferative keratins K6/K16. Keratinocytes in vivo, when exposed to topical retinoids in healthy volunteers, displayed an increase in K13 and K6/16 synthesis, but no alterations in K1/10 and K14 expression. Interestingly, in contrast to normal keratinocytes, in psoriatic skin systemic retinoid treatment seems to reverse the altered enhanced K6/16 in psoriasis, with restoration of suprabasal K1/10 expression.

This illustrates that retinoid response of keratinocytes in vivo and in vitro do not always correlate and that normal keratinocytes respond differently to retinoid than diseased keratinocytes.

In skin (pre) malignancies, as in psoriasis keratinocytes are diseased or dysplastic. The response of the dysplastic or malignant keratinocytes in vivo to retinoid treatment with respect to markers for keratinisation was not studied previously.

In chapter **3.1** we describe the results of a retrospective nonrandomized immunohistochemical study to study the effects of retinoids on the expression of keratin (K)13 and K19 in 21 cutaneous warts of renal transplant recipients (RTRs) and immunocompetent individuals (ICIs).

Nine RTRs (10 specimens) received either systemic acitretin or topical all-trans-retinoic acid treatment at the time of biopsy. A significantly higher percentage of warts of RTRs expressed K13 compared with warts of ICIs (86% vs. 14%, respectively; $P< 0.001$). In warts of RTRs, retinoid treatment correlated significantly with a particularly strong, segmental K13 expression pattern, which we termed zebroid. Without use of retinoids, K13 was mostly restricted to suprabasal single cells. Keratin 19 was absent in all warts of both patient groups.

Since retinoids strongly correlated with K13 in a characteristic zebroid pattern in warts of RTRs, we concluded that K13 is a sensitive marker for retinoid bioactivity in skin (lesions) of RTRs.

In non-retinoid-treated RTRs, K13 is also frequently found in warts, but without the dramatic zebroid pattern noted with retinoid-treated warts. Maybe this single cell expression of K13 in

warts of RTRs has a different biological meaning and is indicative of a more malignant phenotype. Thus far, immunohistochemical studies in skin (pre)malignancies indicated that cutaneous malignant transformation was heralded by a switch from production of larger MW keratins normally present in the epidermis (K1/10) to smaller MW keratins (K8,18 and 19) characteristic of fetal skin and simple epithelia and that the extent of shift correlates with the histological grade of the tumor. K13 expression was reported to be absent in (pre)malignant epidermal tumors. In the study reported in 3.1 we also studied 20 cases of squamous cell carcinoma in situ (SCCIS or Bowen's disease) and we found K13 expression in 75% and 45% of lesions in RTRs and ICIs, respectively. This further supports that K13 expression also might reflect presence of a more malignant phenotype. In these SCCIS the perilesional skin in 3 of 4 retinoid treated RTRs the same zebroid K13 expression as in retinoid treated warts. This retinoid related zebroid K13 pattern was clearly different and distinguishable also in SCCIS.

In chapter **3.2** we describe the results of a histological and immunohistochemical study regarding the effects of systemic acitretin treatment on actinic keratoses in 33 RTRs. Following acitretin treatment, a significant reduction in epidermal thickness (P= .002) and a significant increase in normal differentiation parameter K10 (P= .02) was observed. After acitretin treatment, an increase in K13 (P= .006) and K19 (P= .05) was found. This K13 expression was located suprabasal and band like (zebroid), while K19 was expressed in the basal epidermal layer. As in the study in warts in chapter 3.1 expression of K13 and K19 were uncoupled. Epidermal proliferation did not change, nor did apoptosis, inflammation, keratinocytic epidermal neoplasia score, or transglutaminase staining. At baseline in 8 actinic keratoses a single cell expression pattern of K13 and/or K19 was found, correlated with higher epidermal proliferation (Ki-67). Baseline K13 expression also correlated with higher incidence of SCCs before retinoid treatment. These findings with respect to spontaneous (non-retinoid-induced) K13 expression in AKs, could in analogue to spontaneous K13 expression in warts of RTRs, be a reflection of the more malignant phenotype of AKs in RTRs.

It was concluded that acitretin improves the aspect of actinic keratoses via alteration of keratinization, resulting in peeling of the stratum corneum. No significant change in proliferation was found, which may explain for the rapid recurrence of actinic keratoses seen after cessation of acitretin treatment.

The accompanying randomized clinical study [45] (not published in this thesis), 26 RTRs were treated with oral acitretin during 1 year, demonstrated a significant improvement of AKs with respect to thickness of the lesions ($p < 0.01$). In addition there was an almost 50% reduction in the number of AKs , while the number of malignant tumors was comparable to the number in the year before the study.

In chapter **3.3** a prospective non-randomized clinical and immunohistochemical study was performed to evaluate effects of temporary cessation of systemic acitretin treatment in renal transplant recipients (RTRs). We used K13 as a marker for retinoid bioactivity based on the findings in 3.1 and evaluated effects of acitretin cessation on expression of cell-cycle associated proteins MIB-1, p53 and p16. Previous studies reported a temporarily effect of retinoid treatment with a rebound and increase in skin tumors after cessation of systemic retinoid treatment. Therefore the number of (pre)cancers before, during and after treatment were compared. Since retinoid treatment has considerable side-effects, this stop-study with the help of K13, might help in establishing an optimal interval for intermittent retinoid treatment. Nine RTRs on systemic acitretin (mean duration 2.3±0.6 yrs; mean dosage 18.3±9.9mg) were asked to interrupt their treatment for three months. Patients gave written informed consent. Patients were seen at intervals of 6 weeks, starting at the moment of acitretin interruption. Each visit epidermal lesions were counted; erythema and desquamation

of normal skin and AK were recorded and induration of AKs, a visual analogue score (VAS) for patient's contentedness was performed, and clinical comparable AKs were biopsied.
After 6 and 12 weeks, a significant decrease of K13 expression was found (p=0.03 and p=0.02 respectively); zebroid K13 expression also decreased but this reduction was not significant (5/9 cases at the start vs. 1/9 cases after 6 and 12 weeks ; p=0.10). Cessation of acitretin treatment had no influence on expression of the markers MIB-1, p53, and p16. These results suggest that retinoids exert their effect by alteration of differentiation without effects on proliferation or effects on tumor suppressor protein expression. The lower percentage of zebroid K13 expression in AKs, 56%, compared to warts, 90%(chapter 3.1), during retinoid treatment might be caused by dysplastic changes in KIN lesions with decrease in retinoid receptors or altered retinoid receptor interactions and/or alterations in keratinocyt gene regulation in dysplastic keratinocytes.
Future studies combining immunohistochemistry for K13 and retinoid receptors could answer this question. Previously it was shown that retinoid receptors decreased with progression of cutaneous tumors. In addition, study of K13 expression within one patient of both benign and (pre)malignant skin lesions during retinoid treatment, can yield information on interlesional differences in retinoid sensitivity of skin lesions.
This "stop"-study demonstrated a significant increase in the number of warts after 3 months of retinoid cessation (p=0.02), but there was no significant increase in number of AKs (p=0.09) or SCCs (p=0.3). This supports the above mentioned assumption that warts or benign skin lesions contain more retinoid receptors when compared to (pre)malignant skin tumors. Patients were significantly less satisfied with the cosmetically aspects of their skin and AKs were more indurated (p=0.02). The latter can readily be explained by increase in hyperkeratosis in the absence of retinoids. The study in 3.1 demonstrated that retinoids act anti-hyperkeratotic by induction of K13 expression, which leads to a non-keratinizing type of squamous differentiation. When retinoids are stopped this antihyperkeratotic effect will diminish.
All three studies indicate that K13 expression can be related to two different and independent mechanisms, dependent on the expression pattern present: zebroid or retinoid-associated keratinisation versus single cell positivity or associated with malignant transformation. In none of the studies a clinical anticarcinogenic effect of retinoids could be demonstrated, although a cosmetic improvement was evident.
Future randomized studies case controlled studies with histology and immunohistochemistry over a longer time period and in large patient groups is needed to elucidate whether retinoid treatment is truly anticarcinogenic.

4.2
Samenvatting en conclusies

Tumorsuppressor eiwitten p53, p16 en p14 in cutane carcinogenese ; invloed van zonexpositie, transplantatie en HPV

Zonexpositie is een belangrijke oorzakelijke factor in het ontstaan van huidkanker, in casu plaveiselcel carcinoom (PCC), en voorstadia hiervan, die we keratinocytaire intraepidermale neoplasie (KIN) noemen. Uit onderzoek is gebleken dat in deze (pre)maligniteiten vaak ultraviolet (UV)-gerelateerde mutaties voorkomen van het p53 tumor-suppressor gen. Meer recent werden ook UV-gerelateerde mutaties aangetoond van het CDKN2A (INK4a-ARF) locus dat codeert voor de tumorsuppressor eiwitten p16 en p14.
In immuuncompetente patiënten bleken KIN laesies en plaveiselcel carcinomen van de huid frequent overexpressie te tonen van de tumorsuppressor eiwitten p53 en p16. Naar p14 expressie in huid(pre)maligniteiten werd vooralsnog nauwelijks onderzoek verricht.
Niertransplantatie-patiënten ontwikkelen frequent huidcarcinomen, met name plaveiselcel carcinomen, die veelal pas een aantal jaren na transplantatie beginnen te ontstaan en veelal vanaf dat moment exponentieel in aantal toenemen. Deze carcinomen worden veelal vooraf gegaan, dan wel begeleid, door toegenomen aantal wratten en hyperkeratosen. Deze laatste blijken histologisch veelal te berusten op KIN laesies. Al deze laesies worden met name gezien op zonblootgestelde lichaamsdelen, komen meer voor bij patiënten in zonnige klimaten, en zijn frequenter bij patiënten meer zonexpositie in het verleden. Dit suggereert ook bij niertransplantatiepatiënten derhalve een grote rol voor zonexpositie bij het ontstaan van huid(pre)maligniteiten. Eerdere studies hebben aangetoond dat ook in plaveiselcel carcinomen van de huid bij niertransplantatiepatiënten UV-gerelateerde p53 mutaties voorkomen (aantal patiënten dat onderzocht werd is veelal beperkt met veel variatie in gevonden frequenties van p53 mutaties, variërend van 8-43%). Ook p53 overexpressie in huid(pre)maligniteiten van deze patiënten is beschreven; soms worden vergelijkbare bevindingen gemeld met de normale populatie, echter een aantal onderzoeken vinden frequenter p53 overexpressie in huidlaesies bij niertransplantatiepatiënten.
Onderzoek naar p16 - en p14 mutaties en -eiwitexpressie in huid(pre)maligniteiten in de specifieke subgroep van niertransplantatiepatiënten ontbreekt dusver (er is slechts 1 studie verricht in patiënten met immuunsuppressie die niet nader omschreven zijn).
Omdat niertransplantatiepatiënten in een versneld tempo en in veel hogere frequentie huidmaligniteiten ontwikkelen in vergelijking tot de normale populatie en deze huidmaligniteiten zich bovendien vaker agressief gedragen, lijken er bij deze patiënten naast zonexpositie nog additionele factoren bij het ontstaan en het beloop van huidtumoren aanwezig. Hierbij moet dan met name gedacht worden aan de immuun suppressie waarbij de gedachte is dat deze leidt tot verminderde tumorafweer en tevens kan leiden tot verhoogde infectiekans met (oncogene) virussen. Hiervoor zou in de huid het humaan papilloma virus (HPV) in aanmerking komen, aangezien de huidtumoren bij deze patiënten klinisch vaak begeleid worden door (gelijktijdig) voorkomen van (door HPV-geinduceerde) wratten en histologisch frequent in huid(pre)maligniteiten van deze patiënten nog virale kenmerken (bekend van HPV zoals koilocytose, en verruceuze bouw) worden herkend.
In baarmoederhalscarcinoom en voorstadia hiervan, is de rol van hoog risico HPV in het ontstaan middels vele onderzoeken reeds vast komen te staan. Hier blijkt integratie van hoog risico HPV-DNA in het gastheergenoom te leiden tot inactivatie van p53 en overexpressie

van p16 en ook p14. De oncoproteinen E6 en E7 van hoog risico HPV typen spelen hierbij een grote rol.
De rol van HPV in het ontstaan van huidcarcinoom is nog onopgehelderd. In tegenstelling tot baarmoederhalscarcinoom, is in huidcarcinoom integratie van HPV zeldzaam. Verder heeft in vitro onderzoek aangetoond dat het oncoproteine E6 van veronderstelde hoogrisico typen HPV voor het ontstaan van huidkanker, zoals epidermodysplasia verruciformis geassocieerde HPV typen, apoptose zou remmen in een p53-onafhankelijke manier namelijk door stimuleren van proteolyse van het anti-apoptotisch eiwit bak.
In **hoofdstuk 2** werd gekeken of er verschillen zijn in effecten van zonexpositie, immuunstatus en HPV bij het ontstaan van huidmaligniteiten in niertransplantatiepatiënten ten opzichte van de normale populatie. Daartoe werden immuunhistochemische studies verricht naar de expressie van p53, p16 en p14 in keratinocytaire intraepidermale neoplasie (KIN) laesies en plaveiselcel carcinomen van niertransplantatiepatiënten en vergeleken met die in normale immuuncompetente personen. Verder was een onderzoeksvraagstelling of moleculair onderzoek naar mutaties behulpzaam is bij het identificeren van een metastaserend huidcarcinoom als een patiënt meerdere primaire huidtumoren heeft.
Het onderzoek in **2.1** toonde aan dat zowel p53 als p16 overexpressie frequent aanwezig is in huid(pre)maligniteiten, in respectievelijk 86% van huidlaesies werd p53 overexpressie gevonden en in 76% p16 overexpressie. Gecombineerde p53/p16 overexpressie was het meest frequent, 64%. P16 en p53 expressie bleken onafhankelijk van elkaar (p = 0 .8). Immuunstatus (wel of geen transplantatie), zonexpositie en histologische diagnose hadden geen invloed op deze onafhankelijkheid.
Het onderzoek in **2.2** toonde P14 expressie in 42 van de 105 (40%) onderzochte huid(pre)maligniteiten. P14 expressie was hierbij veel lastiger te detecteren in coupes dan expressie van p16 en p53, omdat meestal slechts weinig cellen positief waren voor p14. Ook expressie van p14 en p53 en van p14 en p16 bleken onafhankelijk van elkaar. Ook hier had immuunstatus en zonexpositie geen invloed op deze onafhankelijkheid (p=0.1).
Dit suggereert dat de 2 belangrijke paden betrokken bij de controle van de celcyclus, respectievelijk de routing via p16/CDK4-6/pRb en die via p14/MDM2/p53, beide betrokken zijn in cutane carcinogenese en dat bij niertransplantatiepatiënten dezelfde paden betrokken zijn als bij immuuncompetente personen. De hogere snelheid waarmee niertransplantatiepatiënten echter tumoren ontwikkelen zou evenwel toch goed bepaald kunnen worden door hun deficiënte immuunsysteem. Getransplanteerd zijn bleek geassocieerd met p53 expressie in plaveiselcel carcinomen (P = .02): plaveiselcel carcinomen van niertransplantatiepatiënten waren vaker p53 negatief (hoofdstuk 2.1 en 2.2). Dit kan betekenen dat in gevorderde stadia van epidermale neoplasie bij niertransplantatiepatiënten, p53 anders betrokken is dan bij immuuncompetenten, waarbij er mogelijk een rol zou kunnen zijn voor (hoog risico?) HPV (uit cervixcarcinoom is namelijk zoals boven beschreven bekend dat hoog risico HPV inactivatie van p53 geeft).
Verder bleek in de studie in hoofdstuk **2.1** dat hooggradige dysplasieën (Hooggradige KIN laesies, HKINs) vaker p16 positief waren dan laaggradige KIN laesies (LKINs) en plaveiselcel carcinomen ($p \leq 0.03$). En HKINs tonen vaker transepidermale p16 en p53 expressie dan LKINs ($P < .001$). De resultaten tav hoge en transepitheliale p16 overexpressie in hooggradige dysplasie/HKIN zijn vergelijkbaar met bevindingen eerder gemeld in de literatuur, met name tav plaveiselcel carcinoom in situ laesies.
Een verklaring voor de hoge p16 overexpressie in HKIN, zou opregulatie van wild type p16 kunnen zijn of eventueel zoals veelal voor p53 overexpressie geldt p16 mutatie. Eerder relatief beperkt onderzoek naar p16 immuunexpressie en onderliggende moleculaire veranderingen in huid(pre)maligniteiten heeft voor alsnog geen duidelijke correlatie laten zien tussen p16 expressie patronen en onderliggende moleculaire veranderingen. Beperkt

werk dat wij zelf hebben gedaan in de studie van de gemetastaseerde plaveiselcel carcinomen in hoofdstuk **2.3** van dit proefschrift bevestigt de bevindingen in de literatuur. In deze groep van 8 gemetastaseerde plaveiselcel carcinomen van de huid was er geen correlatie aantoonbaar tussen aan/afwezigheid van p53/p16 of p14 mutatie en aan/afwezigheid van immuunexpressie voor deze markers. In 5/8 gevallen was er p53 mutatie aantoonbaar: 3/5 gemuteerde tumoren toonden p53 overexpressie, waarvan 2 sterk (>50% positieve tumorcellen), terwijl 2/5 p53-immuunnegatief waren. P16 mutatie was aanwezig in 5/8 gevallen, waarbij 2 tumoren p16 expressie toonden (> 10% tumorcellen positief), echter 3 tumoren bleken p16 negatief. Van de 3 p14-gemuteerde tumoren, was 1 sterk positief voor p14, echter 2 tumoren waren p14 negatief (data verder niet beschreven).
Toekomstig onderzoek naar de exacte rol van deze tumor suppressor eiwitten in huidmaligniteiten zou meer licht op expressie patronen en onderliggende moleculaire veranderingen kunnen werpen. O.a. zou het interessant zijn te onderzoeken wat de rol van p16 is bij overgang van HKIN/plaveiselcel carcinoom in situ naar invasief plaveiselcel carcinoom, aangezien hier weer afname van p16 overexpressie wordt gezien.
In hoofdstuk **2.3** bleek verder dat de frequentie van p16 mutaties in naar de lymfklier gemetastaseerd plaveiselcel carcinoom van de huid zeer hoog is in vergelijking tot frequenties in de normale populatie, zij het dat onze onderzoeksgroep wel beperkt was (n=8). In de gemetastaseerde carcinomen vonden wij een frequentie van 63% p16 mutaties, terwijl gerapporteerde frequenties van p16 mutaties in de literatuur in sporadische niet gemetastaseerde huidcarcinomen variëren van 15-19%. Aanvullende klinisch-pathologische studies naar het voorkomen van p16 mutaties in gemetastaseerde versus niet-gemetastaseerde in grotere patiënten groepen zou zinvol zijn. Mogelijk is p16 mutatie een predictor voor meer agressief beloop en hogere metastaseringskans.
Hoofdstuk **2.3** wordt aangetoond dat mutatieanalyse van p53 en INK4a-ARF behulpzaam is in de identificatie van primaire huidtumoren in geval van metastasering, aangezien de mutatiefrequentie van beide genen in (gemetastaseerde) huidcarcinomen hoog is, namelijk 88% (7/8 gevallen). In 6/7 (86%) gevallen waren wij in staat met zekerheid de primaire tumor vast te stellen die verantwoordelijk was voor de metastase. In de toekomst zou dit type mutatieanalyse bij patiënten met multipele huidtumoren een goede bijdrage kunnen leveren aan zekere identificatie van primaire tumoren, waarna aanvullende vergelijkend onderzoek (histopathologisch, klinisch, moleculair, array technieken) tussen wel en niet gemetastaseerde tumoren, risicofactoren c.q. een risicoprofiel voor metastaserend gedrag zou kunnen opleveren. Deze studie toont overigens ook het belang van goede histologische bewerking met invriezen van tumormateriaal op de afdeling pathologie. In 6/14 gevallen was geen gevroren tumorweefsel aanwezig en bleek paraffine ingebed materiaal van onvoldoende DNA kwaliteit om PCR reacties te verrichten.
In hoofdstuk **2.2** werd tot slot nog HPV detectie verricht op de 105 huid(pre)maligniteiten waarop tevens immuunexpressie van p14, p16 en p53 was bepaald. De HPV bepaling werd verricht op aansluitende weefselcoupes met behulp van een short PCR fragment (SPF-LiPA) assay, waarbij tegelijkertijd 25 mucosale HPV typen gedetecteerd en getypeerd kunnen worden. In slechts 2 van de 105 gevallen werd HPV gevonden; beide gevallen betrof het een HKIN laesie van een immuuncompetent persoon; in beide gevallen betrof het een HPV type X, en bleek het geen bekend mucosaal type HPV te zijn. Helaas kon door dit lage aantal HPV positieve casus geen relatie tussen aanwezigheid van HPV en eiwitexpressie van p14, p15 en p53 bestudeerd worden. Uit dit onderzoek concluderen wij wel dat onze data geen aanwijzing bieden dat mucosale HPV typen een rol spelen bij cutane carcinogenese. Toekomstig onderzoek met inclusie van cutane HPV typen en met name Epidermodysplasia verruciformis geassocieerde HPV typen zou interessant zijn, zeker ook in relatie tot de expressie van de 3

tumor suppressor eiwitten p53, p16 en p14 aangevuld met moleculair onderzoek zoals mutatieanalyse en onderzoek naar promotor methylering.

Retinoid behandeling van benigne en (pre)maligne epidermale tumoren bij niertransplantatiepatiënten

Diverse studies hebben een gunstig effect beschreven van systemische retinoiden behandeling met acitretine of etrenitaat, in de preventie van huid(pre)maligniteiten bij niertransplantatiepatiënten. Systemische behandeling is met name in deze groep van niertransplantatiepatiënten een belangrijke therapievorm, in verband met het grote aantal (pre)maligniteiten in deze patiënten groep. Een aantal studies geven aan dat het effect van deze behandeling wel tijdelijk is met wederom toename van huidtumoren na het stoppen van deze therapie.
In geen van deze studies met systemische retinoidbehandeling bij niertransplantatiepatiënten werd histologisch onderzoek verricht van (peri)lesionale huid, dan wel immuunhistochemisch onderzoek verricht naar markers voor keratinisatie, proliferatie (MIB-1/Ki-67) of markers voor apoptose en cel-cyclus-geassocieerde eiwitten (p53, p16) ten einde meer zicht te krijgen op het chemopreventieve werkingsmechanisme van retinoiden.
Aangezien retinoiden ontstaan van huidkanker en voorstadia hiervan zouden remmen, lijkt het aannemelijk dat zij invloed hebben op de differentiatie en/of proliferatie snelheid van keratinocyten en/of op de mate waarin keratinocyten in apoptose gaan.
Uit dierexperimenteel onderzoek en in vitro studies is reeds bekend dat retinoiden keratinisatie en differentiatie van keratinocyten beïnvloeden, waarbij keratines die gezien worden tijdens normale epidermale differentiatie, namelijk K1 en K10, worden onderdrukt en embryonale keratines, K13 en K19 worden geïnduceerd; ook expressie van hyperproliferatieve keratines K6/16 nam toe in keratinocyt-kweken. In vivo onderzoek in gezonde vrijwilligers met topicale retinoiden liet ook een toename van K13 en K6/16 expressie zien. Bij psoriasispatiënten werd echter afname van K6/16 gezien en herstel van normale keratines gezien. Derhalve lijkt de respons op retinoiden van keratinocyten in vitro en in vivo te variëren en afhankelijk te zijn van de toestand waarin deze keratinocyten verkeren (normale/zieke keratinocyten). Ook in (pre)maligniteiten hebben we te maken met afwijkende cq dysplastische/maligne keratinocyten en de respons van deze dysplastische en maligne cellen in vivo op retinoiden tav keratinisatie is voor alsnog niet eerder immuunhistochemisch onderzocht.
In hoofdstuk **3.1** staan de resultaten beschreven van een retrospectieve niet gerandomiseerde immuunhistochemische studie naar de effecten van retinoiden op de expressie van keratines 13 en 19 in 21 wratten van niertransplantatiepatiënten en immuuncompetente individuen. Van de niertransplantatiepatiënten gebruikten 9 patiënten systemische of lokale retinoidtherapie ten tijde van het biopt. In de wratten afkomstig van niertransplantatiepatiënten werd een significant hoger percentage K13 positieve wratten gezien (86%) t.o.v. de wratten in de normale populatie (14%, $P<0.001$). Hierbij lieten de wratten in geval van retinoidtherapie een typisch zebroid patroon zien met sterke K13 expressie in segmentale positieve suprabasale kolommen. Wratten waarbij geen retinoiden therapie was gebruikt toonden alleen positiviteit in suprabasale solitaire cellen (single cell expressie). Keratine K19 expressie werd in geen enkele wrat van beide groepen gezien, ook niet na retinoidbehandeling. I.v.m. de sterke correlatie tussen zebroide K13 expressie en retinoidtherapie, blijkt uit deze studie dat K13 als goede marker te gebruiken is voor de biologische activiteit van retinoiden in de huid(laesies) van niertransplantatiepatiënten. Mogelijk heeft de meer frequente single cell expressie van K13 in niet-retinoid behandelde wratten van niertransplantatie patiënten ook een biologische betekenis: mogelijk duidt dit op een meer maligne fenotype. Uit eerder onderzoek is namelijk gebleken dat bij maligne transformatie in cutane carcinogenese, er een switch plaats vind van hoogmoleculaire

keratines die normaal in de epidermis voorkomen (K1/10) naar laagmoleculaire keratines. K13 is een laagmoleculair keratine; echter aanwezigheid van K13 in (pre)maligne huidlaesies was tot nu toe niet eerder beschreven. In deze zelfde studie onderzochten wij ook 20 plaveiselcel carcinoom in situ (M.Bowen) laesies in beide patiënten groepen en vonden wel degelijk K13 expressie in 75% en 45% van deze laesies bij respectievelijk de niertransplantatiepatiënten en immuuncompetenten. Dit steunt de hypothese van een meer maligne fenotype van wratten bij niertransplantatiepatiënten. Bij deze carcinoom in situ laesies toonde de perilesionale huid in 3 van de 4 met retinoiden-behandelde niertransplantatiepatiënten ook zebroide K13 expressie. Het retinoid-geassocieerde K13-patroon bleef duidelijk anders en goed herkenbaar.

In hoofdstuk **3.2** staan de resultaten van een studie naar de histologische en immuunhistochemische effecten van systemische acitretine behandeling op actinische keratosen van 33 niertransplantatiepatiënten. Histologisch werd een significante reductie van epidermale dikte (p= 0.002) gezien, als gevolg van afname van de stratum corneum dikte. Immuunhistochemisch werd alleen significante toename cq herstel van K10 expressie gezien (*P*= 0.02) en inductie van K13 (p=0.006) en K19 (p= 0.05) gezien. De K13 expressie werd suprabasaal gevonden met een meer bandvormig aankleuringspatroon, en de K19 expressie met name basaal. Dus net als in wratten was er ontkoppeling van K13 en K19 expressie. Epidermale proliferatie (Ki-67), apoptose (p53), hyperproliferatie geassocieerde keratine (K16), terminale differentiatie (transglutaminase), en dermale ontsteking bleven onveranderd. Onbehandelde aktinische keratosen toonden deels K13 en K19 expressie echter in een meer single cell expressie patroon, waarbij beiden correleerden met een hogere epidermale proliferatie (Ki-67). Baseline K13 expressie bleek ook te correleren met een hogere plaveiselcelcarcinoom incidentie voor retinoidtherapie. Deze bevindingen omtrent spontane K13 expressie in onbehandelde aktinische keratosen, zouden parallel aan de bovenstaand beschreven spontane (niet-retinoid-geinduceerde) K13 expressie in wratten van nierrecipiënten kunnen duiden op een meer maligne fenotype van de aktinische keratose.

In de bij dit onderzoek horende gerandomiseerde klinische studie [45] (niet in dit proefschrift gepubliceerd), werden 26 nierrecipiënten gedurende 1 jaar behandeld met oraal acitretine, waarbij klinisch een significante verbetering van de actinische keratosen werd gezien tav de dikte van de laesies (*P*<0.01). Tevens was er een klinisch een bijna 50% afname van het aantal aktinische keratosen, maar het aantal maligne tumoren tijdens het jaar van behandeling was gelijk aan het aantal in het jaar vooraf aan de studie.

In hoofdstuk **3.3** staat een prospectieve niet gerandomiseerde immuunhistochemische studie beschreven waarin we met behulp van K13 als marker van retinoidactiviteit (op basis van de studie in 4.1) gekeken hebben of stoppen van retinoidtherapie (systemisch acitretine) invloed had op de expressie van celcyclusgeassocieerde eiwitten MIB-1, p53 en p16. Volgens eerdere gepubliceerde studies zouden de effecten van retinoidtherapie tijdelijk zijn met rebound effect en toename van tumoren na staken van de therapie, die mogelijk ook zijn weerslag heeft op markerexpressie in huidlaesies van patiënten. In verband met bijwerkingen van retinoidtherapie zou intermitterende therapie een goed optie kunnen zijn, en deze studie met gebruik van retinoid-geassocieerde K13 expressie als marker van retinoidactiviteit in de huid zou mogelijk tevens een aanknopingspunt kunnen geven over de lengte van het toe te passen interval van stoppen. Klinische effecten werden gedurende de 3 maanden stop geregistreerd.

Bij 9 nierrecipiënten werden biopten van klinisch vergelijkbare aktinische keratosen genomen bij start van de studie (waarop systemische acitretine behandeling werd stop gezet), en na resp. 6 en 12 weken staken van retinoid therapie.

Deze “stop”-studie toonde aan dat staken van acitretine therapie, leidt tot reductie van abnormale differentiatie in KIN laesies van niertransplantatiepatiënten met significante afname van K13 expressie. Staken van acitretine therapie had geen invloed op expressie van

de markers MIB-1, p53 en p16. Dit suggereert dat retinoiden hun effect hebben middels verandering van differentiatie, zonder proliferatie of tumorsuppressor expressie te beïnvloeden.
Een zebroid K13 patroon werd in 56% van de KIN laesies aangetroffen bij start van de studie met een dalende trend gedurende de 3 maanden studie periode (11%, 1/9 patiënten). Het lagere percentage KIN laesies met zebroide K13 expressie tijdens retinoidtherapie ten opzichte van wratten in de studie in hoofdstuk 4.1 (56% vs. 90%) zou veroorzaakt kunnen worden door dysplastische veranderingen in de KIN laesies met afname van retinoidreceptoren of veranderde receptor interacties en/of veranderingen in keratine gen regulatie in dysplastische keratinocyten.
Toekomstig onderzoek in biopten naar gecombineerde aanwezigheid van (zebroide) K13 expressie en aanwezigheid van retinoidreceptoren in huidlaesies zou hierop mogelijk een antwoord kunnen geven. Eerder onderzoek naar retinoidreceptoren heeft al aangetoond dat deze af zouden nemen bij progressie van cutane tumoren. Ook onderzoek naar bijvoorbeeld K13 expressie binnen 1 patiënt ten tijde van retinoidbehandeling met zowel benigne als (pre) maligne huidlaesies kan zinvolle informatie opleveren over interlesionale verschillen in retinoid gevoeligheid van huidlaesies.
In deze studie werd binnen de 3 maanden therapie stop een significante toename van wratten gezien, maar geen significante toename van (pre)maligne huidlaesies. Dit steunt bovenstaande veronderstelling met meer retinoidreceptoren in wratten ten opzichte van actinische keratosen. Patiënten waren ook significant minder tevreden over de cosmetische aspecten van de huid. Induratie van aktinische keratosen nam toe. Dit kan zeer goed verklaard worden door toename van hyperkeratose zonder retinoiden. Retinoiden werken namelijk door inductie van K13 antihyperkeratotisch doordat ze een niet verhoornende plaveiselcellige differentiatie induceren, en staken van medicatie geeft dan weer toegenomen keratinisatie.
Alle 3 de studies duiden erop dat K13 expressie gerelateerd kan zijn aan twee onafhankelijke mechanismen, afhankelijk van het type expressie patroon: zebroid cq retinoid-geassocieerde keratinisatie versus single cell expressie cq duidend op maligne transformatie.
In geen van de studies kon een evident anticarcinogeen effect van retinoiden worden aangetoond. Wel was er een cosmetische verbetering van de huid onder invloed van retinoidtherapie.
Toekomstige studies, gerandomiseerd en gecontroleerd met histologie en immuunhistochemie, gedurende lange tijd en in grotere groepen van niertransplantatiepatiënten zullen meer uitsluitsel moeten geven omtrent een daadwerkelijk anticarcinogeen effect van retinoidtherapie.

Chapter 5

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List of publications/Publikatielijst
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Curriculum vitae

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5.3
DANKWOORD

Toen ik bijna 10 jaar geleden solliciteerde bij de afdeling Pathologie op de vacature van assistent in opleiding vanuit mijn kraambed, waren er 5 vacatures, waarbij de keuze was voor een zgn. AGIKO constructie, een aanstelling gekoppeld aan promotieonderzoek, ofwel een aanstelling als louter assistent pathologie. De keuze was voor mij toen gemakkelijk, namelijk geen onderzoek voor deze dame. Gaandeweg de opleiding had ik al snel door dat ik een grote voorliefde had voor het gebied van de dermatopathologie. Professor van Haelst was degene die destijds dit vakgebied in het Radboud Ziekenhuis met een zekere haatliefde verhouding bedreef, altijd zeer accuraat beschrijvend en met wel overwogen conclusies. Wetenschappelijk onderzoek kwam voor mij pas later in beeld, aanvankelijk min of meer als must aangezien ik althans deels “academisch” wilde blijven werken. Wat begon als must werd later meer een lust; ik begon het onderzoek leuk te vinden. Het bleek creatiever dan de patiëntenzorg, met innovatieve ideeën die je kon uitwerken, zolang echter de financiële situatie het toeliet, want ik had geen project en moest bij elk ideetje weer ergens wat centen/en later euro’s zien los te peuteren en hopen dat ik analytische ondersteuning kreeg. De eerste 2 jaar heb ik regelmatig zelf met veel plezier trouwens ook in het lab gestaan voor het kleuren van immuno’s en later het doen van PCR reacties. Daardoor heb ik veel respect en bewondering gekregen voor het werk van analisten (die kunnen dit toch echt veel beter dan ik) en denk ik bovendien beter inzicht in het werken van en op zo’n lab.
Bij het tot stand komen van dit proefschrift zijn vele mensen op verschillende manieren van belang geweest. Echter hoewel ik met veel liefde en plezier werk, zal dit toch altijd op de 2e plaats komen en wil ik beginnen met de mensen uit mijn privé omgeving te bedanken en vervolgens alle anderen.

Een van de belangrijkste personen voor mij is natuurlijk mijn vriendman Harold, die altijd als belangrijke spil mede ons gezin met inmiddels een kwartet van kinderen, draaiend houdt. Die daarnaast bovendien altijd degene was die als pispaaltje fungeerde als het weer eens tegenzat en dat wonderbaarlijk genoeg met niet afnemende liefde bleef beantwoorden. Daarnaast leverde hij ook zeker een belangrijke wetenschappelijke bijdrage aan het tot stand komen van dit boekje door altijd mijn artikelen kritisch te lezen en door zijn onmisbare computerondersteuning. Dankzij hem werd ik van een computer nerd, langzaam een iets mindere nerd. Onze kindjes en grote gezamenlijke liefdes Jop (11), Ruth (9), Zoë (6) en recent de afsluiter in de rij Norah (1) zorgden en zorgen voor veel afleiding en de nodige relativiteit tav werk en onderzoek. Als vrouw kan je niet rustig werken als het thuisfront niet goed draait en dankzij onze oppas Door die reeds 10 jaar lang met veel toewijding ons gezin en onze was mede draaiend houdt was en ben ik in staat om te werken zoals ik heb gewerkt en nog vele jaren hoop door te werken.
Pap. Mam, ook jullie stonden altijd klaar als er weer eens geklust moest worden in onze money pitt, of als er weer eens een kinderopvangprobleem was. Datzelfde geldt voor mijn lieve zus Lenneke die zeer veel credit heeft opgebouwd en nog kan rekenen op vele jaren oppas van onze zijde nu ze zelf haar eerste dochter Sam heeft gekregen.
Prof.Dr.D.J.Ruiter, Dirk (mocht ik zeggen vanaf mijn pathologenschap ca 3 jaar geleden waar ik overigens zeer aan moest wennen), mijn promotor, was degene die mij de kans bood om de dermatopathologie op academisch niveau te blijven beoefenen. Als onderzoeker in hart en nieren moest dat wel begeleid worden met een onderzoek en promotie. Jouw werksnelheid is echt bewonderenswaardig, evenals de workload die je kan verzetten en daar heb ik veel

bewondering voor. Niet zeuren, maar doen, hopelijk heb ik dat ook goed van jou mee gekregen. Daarnaast heb je een grote kennis op het gebied van melanocytaire laesies, die ik mezelf nu eigen aan het maken ben met jouw steun sinds jij begin 2004 decaan bent geworden. Jouw kennis op allerlei gebieden is zeer divers, en ook in het onderzoek in dit proefschrift hoewel dat niet primair jouw aandachtsveld was, is deze zeker zeer goed van pas gekomen. Jij zag ook direct het belang van goede samenwerking met de afdeling dermatologie zonder welke dit proefschrift er niet was geweest. Je stimuleert ook de zelfredzaamheid die je in het begin als onderzoeker soms wat hopeloos maakt (uitspraken van jou : dat moet anders, maar hoe ? dat stond er dan niet bij. Of “it’s your baby” wat in mijn geval soms ook letterlijk zo was), maar uiteindelijk ervoor zorgt dat je een vrijwel zelfstandig onderzoeker wordt althans dat hoop ik dat ik ben geworden. Dank voor je expertise op zeer breed gebied.

Prof.Dr.P.C.M. van de Kerkhof, Peter, mijn andere promotor, en in een aantal opzichten tegenpool en daardoor goed tegenwicht van Dirk. Door jouw enthousiasme (“ dit artikel is werkelijk fantastisch”) werd samenwerken met jouw afdeling heel plezierig en constructief niet alleen op het gebied van onderzoek maar ook op het gebied van patiëntenzorg, met een 12 uur bespreking die nu wekelijks goed loopt en een dermatopathologie-stage die goed draait. Ik bewonder ook bijzonder jouw sociale gaven, en de gelijkwaardige manier waarop je met anderen en ook met mij omgaat/ging, waardoor er volgens mij in jullie staf ook zoveel amicaliteit is naast toch ook veel respect voor elkaars kunnen en zijn. Je commentaar op artikelen was altijd snel, kort en bondig en constructief en je was altijd bereid om actief te participeren. Ik weet nog dat je voor het eerste artikel in de Archives over retinoiden in wratten zelf nog een patiënt thuis hebt opgebeld of hij echt geen retinoiden gebruikt had of crèmes en je een heel verhaal over scheerschuim moest aanhoren. Dank je voor je dermatologische kijk en grote expertise, inzet en zeer plezierige samenwerking.

Dr.E.M.G.J. de Jong, Elke, in het begin was onze samenwerking enigszins met horten en stoten in verband met jouw zwangerschap en bijbehorend verlof dat viel vrij kort na de start van ons gezamenlijke onderzoek. Jurgen Smit en Peter van de kerkhof vingen echter samen de gang van zaken goed op en zo konden we toch vrij snel al de eerste resultaten neerschrijven. Later hebben wij de draad samen weer opgepakt en ik vond het erg plezierig dat jij ondanks jouw drukke bezetting altijd tijd probeerde vrij te maken voor zoveel mogelijk een reguliere afspraak om de 1 a 2 weken. Ik vond je als mens bijzonder plezierig in de omgang en je hebt de gave om mensen te sturen zonder erg op de voorgrond te staan. Daarnaast ben je een bijzonder goed en kundig dermatoloog want ook op het vlak van patiëntenzorg kwam ik je natuurlijk vaak tegen. De samenwerking met jou en jullie afdeling heeft voor mij veel meerwaarde gehad en bijgedragen aan mijn toegenomen, zij het nog steeds beperkte kennis van de dermatologie.

Dr. P.C.M. de Wilde, Peter, mijn statistische steun en toeverlaat. Jouw bijdrage aan het tot stand komen van dit proefschrift was aanzienlijk. Jij was een zeer kritisch coauteur (soms bijna te), en jouw zeer secure aanpak bood een goed tegenwicht aan mijn over 1 dag ijs gaan, zodat de stukken die wij samen produceerden uiteindelijk goed gefundeerd, maar toch niet te laat de pers bereikten. Als collega patholoog “next door”, heb ik je ook zeer gewaardeerd en zijn we in de loop der jaren vanuit mijn perspectief althans meer vrienden dan collegae geworden. Dat laatste mede door onze gedeelde liefde voor drank en spijs. Hoewel ik op het gebied van de vinologie ongetwijfeld een heidense barbaar ben in jouw ogen, want de witte wijnen laat ik staan. Hopelijk zullen we in de toekomst veel blijven samenwerken op het gebied van onderzoek en diagnostiek, want samen coupes kijken is iets wat we in de loop der jaren regelmatig deden en waaruit jouw grote kennis van de orale pathologie bleek, waarvan ik graag deelgenoot werd/word. Als tegenprestatie kijk ik dan graag naar naevi of baso’s die de kaakchirurg per ongeluk mee verwijdert.

Dr. J.V. Smit, Jurgen, dankzij de samenwerking met jou kwamen de eerste publicaties snel van de grond. Jij bent inmiddels al toegetreden tot het gezelschap van doktoren middels je mooie proefschrift op 14 april 2003. Gelukkig kon ik daaraan ook een bescheiden bijdrage leveren. Ik vond het leuk om de patiënten poli's die jij deed van de patiënten die deels studieobject waren van dit proefschrift bij te wonen bij de start van mijn onderzoek zodat ik een beter kader kreeg van de huidproblematiek bij niertransplantatiepatiënten. Ik weet dat je nu een zware periode privé doormaakt, maar hopelijk komen jullie daar goed doorheen.
Mijn collega-pathologen in het CWZ, Marcel, Ewout, Carla, Erik, Anne en Ineke wil ik hartelijk bedanken voor de steun en interesse bij het tot stand komen van dit proefschrift. Veel van de artikelen zijn tot stand gekomen tussen de bedrijven door en ook in CWZ tijd heb ik heel wat zinnen op papier gezet. Vanaf september ga ik de efficiëntie uit de periferie echt wel missen, maar hopelijk kan ik er wat van meenemen naar de academie. Jullie collegialiteit hoop ik te behouden ook al is de afstand nu iets groter geworden. Saskia zal naar mijn idee de dermatopathologie in het CWZ zeer adequaat gaan overnemen.
Veel analisten uit het Radboud, maar ook uit het CWZ (hier met name Ingrid en onder toeziend oog van de mijns inziens uitstekende en altijd tot medewerking bereid zijnde hoofdanalist Henk de Haard), hebben bijgedragen door het snijden van coupes en kleuren van deze, waarvoor mijn grote dank. Speciaal wil ik Monique Link bedanken en later Sabine Aalders, die veel van het uitstekende immuunhistochemisch werk hebben verricht voor de diverse publicaties. Dank ook aan Monique voor het bijbrengen van de basics van de immuunhistochemie zodat ik deels mijn eigen coupes kon kleuren. Ook speciale dank aan Marian van Dijk die monnikenwerk heeft verricht bij het tot stand komen van een goed lopende PCR voor INK4a-ARF mutaties. Dank aan Riki Willems voor de leerzame stage op het moleculaire lab. Dat viel erg tegen om zelf te doen. Petje af voor jullie expertise.
Arie Maat dank voor het altijd probleemloos vers verwerken, splitsen en vriezen, van de huidjes voor het onderzoek van Jurgen en mij. En daarnaast ook voor de wekelijkse keek op de week op vrijdagmiddag als je met mij mee terugreed en –rijdt naar ons mooie land van Maas en Waal.
Willem Melchers, dank aan jouw virlogische bijdrage aan het artikel over p14 en HPV, en dank voor de eindsprint die jij nog even hebt ingezet mede op verzoek van Han van Krieken (waarvoor dank Han) zodat ik het manuscript in mijn zwangerschapverlof van Norah kon afronden.
Marjolein Ligtenberg ben ik veel dank verschuldigd bij het tot stand komen van het artikel over p53 en INK4a-ARF mutaties. Dankzij jouw bijdrage werd het uiteindelijk een uitstekende publicatie.
Dank ook aan het alle secretaressen zowel op het CWZ als in het Radboud die voor mij ontelbare coupes en/of blokjes uit het archief hebben gelicht (later hielp ook Wim Jansen hieraan mee) en/of mijn artikelen met veel zorg opstuurden.

5.4
CURRICULUM VITAE

Willeke Blokx werd geboren op 30 juni 1967 te Den Dungen. Van 1979 tot 1985 doorliep zij het Gymnasium Beekvliet te St. Michielsgestel. Na 2 jaar opleiding fysiotherapie aan de S.U.P.A. te Utrecht, ving zij aan met de studie geneeskunde aan de Rijksuniversiteit Utrecht. Zij behaalde haar doctoraalexamen cum laude in 1991. Van 1991 tot 1994 volgde zij haar co-schappen waaronder een keuzeco-schap pathologie in het AZU. Op 29 juli 1994 behaalde zij haar artsexamen. Na de geboorte van haar eerste zoon Jop, begon zij in februari 1995 aan haar opleiding tot patholoog op de afdeling pathologie van het Universitair Medisch Centrum St. Radboud, met als afdelingshoofd en toenmalige opleider Prof.Dr.D.J.Ruiter. In het CWZ te Nijmegen volgde zij van 1-12-1997 tot 1-10-1998 de B-opleiding pathologie, met als opleider Dr.E.Schaafsma. Op 21 november 2000 voltooide zij de opleiding pathologie. Vanaf 1 december 2000 tot en met heden is zij werkzaam voor 2 dagen per week als klinisch patholoog in het CWZ te Nijmegen. Daarnaast werkte zij tezelfdertijd 2 dagen per week in het UMC St.Radboud, eerst vanaf 1 december 2000 tot 1 december 2002 als artsonderzoeker en vanaf 1 december 2002 als staflid bij de vakgroep pathologie. In de academische dagen werkte zij aan de onderzoeken welke geleid hebben tot dit proefschrift met daarnaast verrichten van patiëntenzorg met als aandachtsveld de dermatopathologie. Inmiddels is zij inmiddels tevens moeder van 4 kinderen (Jop, Ruth, Zoë en Norah) en vervult zij naast haar werkzaamheden hierboven samen met haar manvriend Harold van Rijen een zeer gelukkig co-ouderschap.
Per 1 september 2005 zal zij geheel werkzaam worden in het UMC St.Radboud als chef de clinique op de afdeling pathologie, met nog steeds het geliefde aandachtsveld van dermatopathologie en onderzoek op dit gebied.